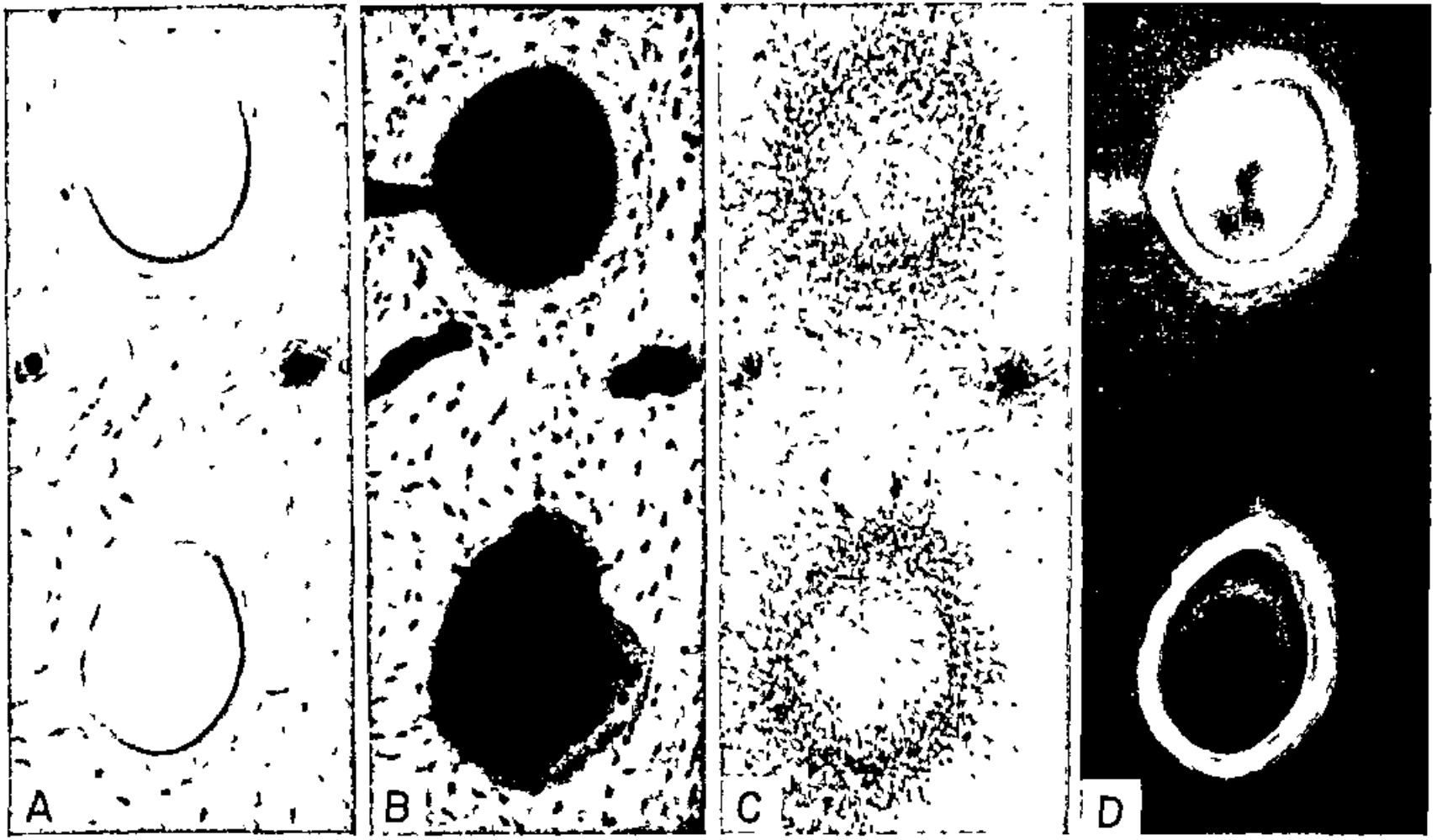

FOUR ILLUSTRATIONS OF THE SAME SECTION OF COMPACT BONE FROM THE RADIUS OF A DOG

The bone is from an 8-month-old beagle, given tetracycline, 25 mg/kg, 4 days and Ra^{226}, 10 μc, 2 days before sacrifice, both intravenously. The bone was embedded in methyl methacrylate and the section, 100 μ thick, was cut with a high-speed rotary saw, without further treatment. *A*, photomicrograph of unstained section. *B*, microradiograph, made with 9 KV X rays on Eastman Kodak 649–0 spectroscopic plate. *C*, alpha-track autoradiograph, made with stripping film, Kodak Limited AR–10, exposed for 14 days. *D*, fluorescent image with ultra-violet light, photographed on Kodak Panatomic X film. All images × 180. (Reproduced by courtesy of R. E. Rowland, Argonne National Laboratory.)

BONE

An Introduction to the Physiology of Skeletal Tissue

SECOND EDITION
REVISED AND ENLARGED

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Preface to the Series

During the past few decades the investigative approaches to biological problems have become markedly diversified. This diversification has been caused in part by the introduction of methods from other fields, such as mathematics, physics, and chemistry, and in part has been brought about by the formulation of new problems within biology. At the same time, the quantity of scientific production and publication has increased. Under these circumstances, the biologist has to focus his attention more and more exclusively on his own field of interest. This specialization, effective as it is in the pursuit of individual problems, requiring ability and knowledge didactically unrelated to biology, is detrimental to a broad understanding of the current aspects of biology as a whole, without which conceptual progress is difficult.

The purpose of "The Scientist's Library: Biology and Medicine" series is to provide authoritative information about the growth and status of various subjects in such a fashion that the individual books may be read with profit not only by the specialist but also by those whose interests lie in other fields. The topics for the series have been selected as representative of active fields of science, especially those that have developed markedly in recent years as the result of new methods and new discoveries.

The textual approach is somewhat different from that ordinarily used by the specialist. The authors have been asked to emphasize introductory concepts and problems, and the present status of their subjects, and to clarify terminology and methods of approach instead of limiting themselves to detailed accounts of current factual knowledge. The authors have also been asked to assume a com-

Foreword

As recorded in the Foreword to the First Edition of this book, its origin goes back to our association with A. Baird Hastings and later with William Bloom, beginning thirty years ago. It was largely owing to their stimulus and guidance that our interest was directed to calcium metabolism and that this led to the formulation of our concepts of the physiology of bone.

In six years since the First Edition was published there has been rapid progress in many aspects of the physiology and biochemistry of bone, as there has been in all other branches of medical science. In reflecting this progress the Second Edition represents an extensive revision, amounting, in the greater part of the book, to a complete rewriting. The previous organization, however, has for the most part been retained; the principal changes in chapter headings represent increased emphasis on regulatory processes.

Acknowledgment is again made to the Josiah Macy, Jr. Foundation, which not only supported our work by grants-in-aid over much of the period from 1933 to 1960, but also influenced its direction through the Macy Foundation Conferences relating to its subject, from 1941 to 1953. We are greatly indebted to Frank Fremont-Smith, formerly medical director of the Macy Foundation, for his unfailing interest and encouragement, which still continue.

Currently, work in our laboratories is being supported by the Division of Research Grants, National Institutes of Health, United States Public Health Service (Grants Nos. A–4225 and A–3793, respectively). The United States Atomic Energy Commission has also contributed by a grant to the University of Chicago (AT

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CHAPTER I

Introduction

By whatever pathway one comes to the study of bone, one finds one's self in the midst of a complex system of structure and function on the macroscopic, microscopic, and ultramicroscopic levels, with numerous and varied chemical and physiologic interrelations. These furnish the subject matter of the physiology and biochemistry of bone, to which this is directed. The approach is multidisciplinary. Morphology, experimental embryology, histochemistry, enzyme chemistry, crystallography, electron microscopy—to mention only a few—have all been brought to bear on the problems of bone.

This book is concerned with bone as a tissue rather than with its mechanical function in providing the skeletal support of the body or with the shape, adaptation, or development of individual bones. Bone, far from being passive and inert, has a complicated physiology of its own and presents a series of challenges to all the medical sciences. The intent has been to bridge the gaps remaining between the various disciplines and to contribute to the unification of the subject. That this requires the use of the vocabularies of many branches of science is inevitable.

This is not intended as a full-scale monograph on the physiology of bone. Historical treatment is reduced to a minimum; documentation is limited; illustrations are few. On the other hand, the text provides an extended treatment of the current state of the literature. The authors are naturally most familiar with their own work and that of their collaborators, but these contributions have not been overemphasized.

While there is a logical sequence in the arrangement of the chapters and the material has been developed accordingly, each

*is intended to stand by itself, to be read and understood independ*ently by those especially interested. To this end there is a minimum of cross-references between chapters; where the need for these is felt, the Table of Contents should serve the purpose. This method of treatment necessarily results in a certain amount of repetition; where the same subject appears in different chapters, the attempt has been to furnish a fresh point of view in each instance.

This book is to complement the many clinical treatises on bone. Few of the pathologic conditions of unknown etiology and none of the tumors of bone are considered here. Disorders of metabolism are treated from the standpoint of pathologic physiology rather than of diagnosis and treatment. The chapter on healing of fractures presents knowledge that can be applied to practical use in clinical work. It is incidental, however, to the results of research on the physiology of bone, a field of endeavor in its own right. This book is written for an audience to include those desiring both a broad acquaintance with the skeletal system and a deep insight into its fundamental problems.

CHAPTER II

Bone as a Tissue

Bone is a highly specialized form of connective tissue, composed of branching cells in an intercellular substance and forming the skeleton or framework of the body of most vertebrates. There are certain characteristics that differentiate it from other forms of connective tissue, of which there are many types, the most striking one being that it is hard. This hardness results from deposition, within a soft organic matrix, of a complex mineral substance, composed chiefly of calcium, phosphate, carbonate, and citrate. Bone has cells peculiar to it; they are specialized forms derived from cells common to connective tissue.

The interstitial substance, in addition to being calcified, has a fibrillar structure similar to that of connective tissue; the fibers are mainly those of collagen; reticular fibers have also been demonstrated. The ground substance, as in connective tissue, is characterized in bone by its content of mucopolysaccharides. Connective tissue and bone have in common the important function of the support of organs or elements of organs. The mechanical and physiologic functions of both tissues are inseparable.

Bone is also closely related to cartilage, another specialized form of connective tissue. Most of the embryonic skeleton is laid down first as models of hyaline cartilage, the cells of which hypertrophy and undergo changes in their chemical characteristics immediately prior to their replacement by bone. A portion of the cartilage matrix remains, is calcified, and serves as the cores of trabeculae of bone. Following a fracture of a bone, initially preformed in cartilage, cartilage and fibrocartilage appear in the first attempt at repair—the callus. In certain locations either tendon or calcified cartilage may be incorporated within bone and may undergo direct transformation to bone tissue.

CELLS OF BONE

The cellular components of bone are associated with specific functions: *osteoblasts* with the formation of bone; *osteocytes* with the maintenance of bone as a living tissue; and *osteoclasts* with the resorption of bone. These cells, having common ancestors, are closely interrelated. During active growth, frequent transformations occur from one to another of the morphologically different forms, while they retain the potencies common to all three forms; the morphology of a particular cell at any given time depends upon the function it is being called upon to perform. The transformations from one type to another, occurring spontaneously more frequently in developing bone, can be demonstrated in adult bone under certain conditions. The most striking examples of such responses are seen during the healing of fractures, in hyperparathyroidism, or in the bird or mouse under the influence of estrogens.

Osteoblasts. These appear on the surface of a growing or developing bone. During active growth they appear to be in a continuous layer, frequently connected with one another by thin cytoplasmic processes. They are cuboidal in shape, with a breadth of 15–20 microns. The cytoplasm is intensely basophilic, owing to its content of ribose nucleic acid. The typical morphologic characteristics are absent or unrecognizable when the particular structure with which they have been associated is in a resting state. The osteoblasts then become spindle-shaped and resemble fibroblasts or reticular cells.

With histochemical techniques, the presence of granules staining by the Hotchkiss procedure (periodic acid–Schiff reaction; PAS-positive) can be demonstrated in the osteoblast when new bone is forming These granules disappear when the osteoblast assumes the spindle form. Alkaline phosphatase is present in the cytoplasm of the osteoblast; the intensity depends largely upon the state of development and the functional activity of the cell.

Osteocytes. The osteocyte is an osteoblast that has been surrounded by calcified interstitial substance. The cells are now inclosed within lacunae, and the cytoplasmic processes extend through apertures of the lacunae into canalicules in the bone. Like the osteoblast, the osteocyte undergoes transformations; it may

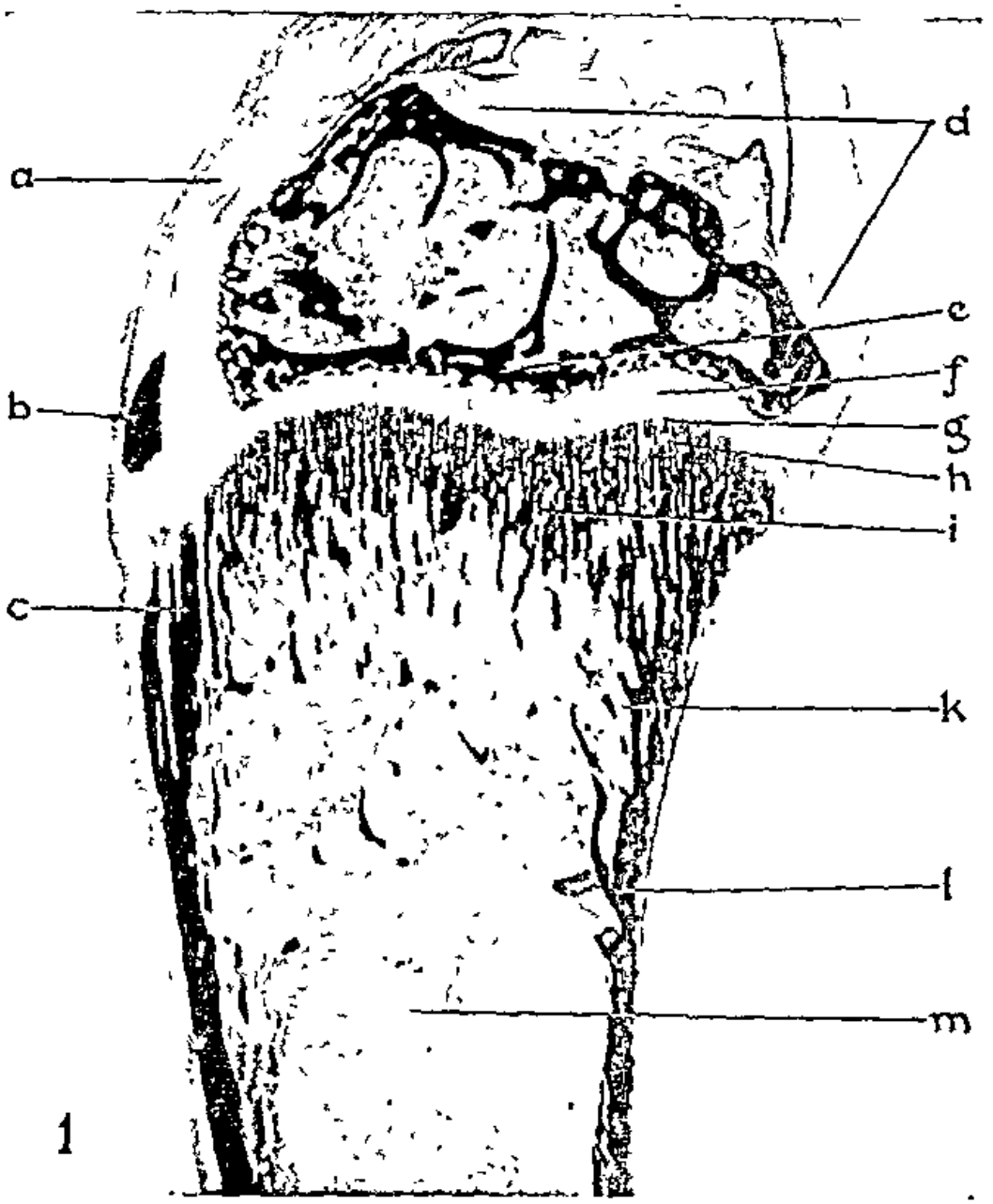


FIG. 1.—Sagittal section of head of tibia of normal rat Cut, without decalcification, by method of McLean and Bloom and stained with silver nitrate (von Kóssa) to illustrate distribution of calcified tissues Age 7 weeks, weaned to Bills's diet at 3 weeks. (*a*) Patellar tendon, (*b*) ossification center of anterior tibial tubercle; (*c*) intratendinous ossification in insertion of patellar tendon; (*d*) insertions of crucial ligaments; (*e*) area in epiphysis where osteoid is frequently seen, (*f*) epiphyseal cartilage plate, (*g*) zone of hypertrophic cartilage and of provisional calcification; (*h*) primary spongiosa; (*i*) secondary spongiosa, (*k*) area at junction of spongiosa with shaft, where osteoid is frequently seen; (*l*) shaft, (*m*) bone marrow Silver nitrate–hematoxylin-eosin. ×16. (From original of Pl. 1, Fig. 1, McLean and Bloom, Anat. Rec., 78:355. Reproduced by courtesy of the publishers)

assume the form of an osteoclast or of a reticular cell. PAS-positive granules have also been demonstrated in osteocytes; those in newly formed bone matrix contain many granules, whereas those in quiescent bone have few. The osteocytes are rich in glycogen; the cytoplasm is faintly basophilic.

Osteoclasts. The osteoclast is a giant cell with a variable number of nuclei, often as many as fifteen or twenty. The nuclei resemble those of osteoblasts and osteocytes; the cytoplasm is often foamy; the cell frequently has extensile processes. These cells may arise from the stromal cells of the bone marrow, or they may represent fused osteoblasts and may also include fused osteocytes liberated from resorbing bone. They are usually found on the surfaces of bone, in close relationship to areas of resorption, and frequently lie in grooves, known as Howship's lacunae. This suggests that the lacunae were formed by an erosive action of the overlying osteoclasts. For many years the osteoclasts have been considered to play the principal role in the resorption of bone. In the growth and re-formation of trabeculae of spongy bone in rapidly growing animals they are commonly seen enveloping the tip of each spicule of bone undergoing resorption.

PAS-positive granules have been demonstrated in osteoclasts and are indistinguishable from those observed in osteoblasts and osteocytes. A brush border, appearing in fixed preparations between the osteoclast and the underlying surface of bone, and long a subject of controversy, now is interpreted to correspond with a very active ruffled and undulating membrane, seen in time-lapse motion pictures of bone undergoing resorption in tissue culture. As in the case of the osteoblast or osteocyte, the osteoclast also can transform into reticular cells.

INTERSTITIAL SUBSTANCE

The intercellular portion of bone *is a calcified collagenous substance* that makes up the great mass of bone. In ordinary sections this appears to be homogeneous, but with special techniques and staining methods collagenous fibers may be demonstrated. The interstitial substance includes the organic framework or matrix,

the inorganic part or the mineral of bone, and water. The organic matrix has two chief components—the collagenous fibers and the ground substance.

MEMBRANES OF BONE

Periosteum. The connective tissue surrounding bone is the periosteum. In the young animal, especially in the regions of rapid growth, this consists of an outer dense layer of collagenous fibers and fibroblasts and an inner looser layer of osteoblasts and their precursor cells. In the quiescent state, in the adult, the periosteum serves for the attachment of tendons and carries blood vessels, lymphatics, and nerves. The inner layer retains its osteogenetic potency and in fractures is activated to form osteoblasts and new bone.

Endosteum. The endosteum is a thin layer of reticular cells, lining the walls of the bone marrow cavities and of the haversian canals of compact bone and covering trabeculae of cancellous bone. It is a condensed peripheral layer of the stroma of the bone marrow; it has both osteogenetic and hemopoietic potencies, and, like the periosteum, it takes an active part in the healing of fractures.

BONE MARROW

Many of the cellular elements seen in loose connective tissue are absent from bone tissue, but their counterparts are seen in numbers in the stroma of the bone marrow. The bone marrow, although more commonly thought of in relation to its hemopoietic functions, also participates actively in osteogenesis. The reticular cells of the stroma of the bone marrow can display osteogenetic activity and undergo transformation into the cells of bone. Similarly, the cells characteristic of bone, when no longer called upon to perform their specific functions, may disappear into the stroma as reticular cells.

MAST CELLS IN BONE

Mast cells are normally found in large numbers in bone marrow and loose connective tissue throughout the body. Their large cytoplasmic granules stain metachromatically with toluidine blue or

azure. They are generally associated with storage of heparin, histamine, and hyaluronic acid. Mast cells can be made to accumulate in the bones of rats by feeding a diet deficient in calcium and in vitamin D or by other conditions of stress. These cells gather on the surface of, within, or under the endosteum when the growth of the animal comes to a standstill (Fig. 2).

BLOOD VESSELS OF BONE

The vascular anatomy of the skeleton has a characteristic pattern closely related to the functions of bone. The blood supply of

FIG. 2.—Sagittal section of upper end of tibia of a rat, 8 weeks of age, reared on a calcium-deficient diet for four and one-half weeks. Deeply staining mast cells within and upon the endosteal lining of bone trabeculae of spongiosa. Hematoxylin-eosin-azure II stain. ×160.

a long bone comes from three sources: (1) the nutrient artery, (2) the periosteal arteries, and (3) the epiphyseal arteries. The nutrient artery perforates the bone obliquely through the shaft and divides into ascending and descending branches. Each branch arborizes to reach the endosteum, the metaphyses, and the epiphyseal plates.

The periosteum has a rich blood supply, consisting of small arteries and veins, forming a continuous vascular network that ensheaths the bone. The number of periosteal vessels is relatively small in the center of the shaft but adds up to hundreds at the metaphyseal ends of the bone. They are of large caliber and anastomose with the cortical branches of the nutrient arteries. The periosteal blood supply is greatly increased when the medullary arterial supply is damaged. The medullary blood supply may increase when the periosteal supply is injured; this represents the collateral route.

The epiphyses are supplied by one to three main epiphyseal arteries, each coursing toward the center and then branching outward to supply a specific area of the spongiosa and articular cortex. The epiphyseal plate is supplied by blood vessels from three sources: (1) perforating epiphyseal, (2) perforating metaphyseal, and (3) circumferential epiphyseal arteries.

The total blood supply to individual bones, together with the corresponding venous drainage, is readily studied by existing methods; the differences in the descriptions in the literature are largely attributable to species differences and to differences in the patterns exhibited by different bones in the skeleton of the same animal. There are, however, unresolved problems, especially those related to the circulation in the haversian canals of compact bone.

Except for an occasional blind ending, each haversian canal contains one or more blood vessels. Variations in the number from one to four are described, with some disagreement as to whether the number is commonly one, two, or three; again, species differences are not fully taken into account. There is agreement that, for the most part, the vessels are small and thin-walled; arterioles, recognizable by smooth muscle in the walls, are unusual. There is no agreement as to whether the thin-walled vessels should be called

venules, capillaries, or sinusoids; the term arteriole is excluded by the morphologic characteristics.

The blood vessels of the haversian canals have rarely, if ever, been traced from their origins to their terminations. In spite of their thin walls, however, and especially when there is only one vessel in a canal, it must be assumed that they are supplied with

Fig 3.—Cross-section through the mid-shaft of a human tibia, injected with India ink and cleared by the Spalteholz technique. Radial branches of the nutrient artery ramify in the cortex to supply the vessels of the haversian canals. ×5.25. (From original of Fig. 3-B, Nelson, Kelly, Peterson, and Janes, J. Bone & Joint Surg., 42A:629. Reproduced by courtesy of the publishers)

arterial blood, and that they drain into the venous system. Functionally, therefore, the vessels are to be regarded as the equivalent of capillaries, since they form the only pathways by which exchange of fluid and dissolved substances occurs between blood and bone. Their transverse branches are found in Volkmann's canals, which represent their horizontal components and afford communication with larger blood vessels (Fig. 3).

NERVES OF BONE

Combinations of morphologic and physiologic preparations are necessary to demonstrate the nerves of bone. The probability is

that there are afferent nerves in the interior of a bone, as well as in the periosteum and endosteum. There is little doubt about the periosteum; it contains abundant myelinated and non-myelinated nerve fibers ending in networks on the surfaces of the bone tissue. Some observers also see myelinated and non-myelinated nerve fibers accompanying the blood vessels in the haversian canals. Myelinated nerves are numerous in the marrow and terminate in the endosteum as delicate fibrils running along the blood vessels.

LYMPHATICS OF BONE

Lymphatic vessels in bone are more difficult to demonstrate by ordinary morphologic techniques than in any other tissue. Particulate matter may be seen in lymphatic vessels in the periosteum, bone marrow, macrophages, and regional lymph nodes within a few minutes after local instillation of India ink or dyes. However, the lymphatic vessels in the marrow-vascular-cell spaces of compact bone cannot be demonstrated by such means. Failure to demonstrate lymphatic vessels in either compact bone or bone marrow has been reported.

CHAPTER III

Histogenesis and Organization of Bone

Bone always arises, both in fetal and in postfetal life, by a transformation of connective tissue. Connective tissue is present throughout the body and assumes many forms. We are concerned with the connective tissue cells that may give rise to the cells of bone and with the interrelations between the cells of connective tissue and those of bone. For an understanding of these relationships there are required: (1) criteria for identification and designation of connective tissue cells, particularly those related to bone and its formation; and (2) a clear and consistent terminology for those cells to which reference is to be make throughout this book.

CRITERIA FOR DESIGNATION OF CONNECTIVE TISSUE CELLS

Below are indicated the criteria we have adopted for classification of these cells, together with the designations we have employed.

Morphologic Criteria. Cells found in loose connective tissue—fibroblasts, macrophages, lymphoid wandering cells, mast cells, and plasma cells—are recognizable under the microscope by their morphology and staining reactions alone. In certain other locations, as in tumors, inflammatory tissue, and perivascular connective tissue, recognition of *fibroblasts* may be difficult; complete characterization of these cells may require criteria other than morphologic. In the absence of definitive criteria, the use of purely descriptive terms, such as *spindle cells, spindle-shaped cells, or fibroblast-like cells,* is common.

Criteria of Location. Many cells, resembling fibroblasts in mor-

phology, may be identified by their locations. Thus the outstretched cells of reticular connective tissue, including the stroma of the bone marrow, are known as *reticular cells.* Among the *perivascular connective tissue cells* are those with mesenchymal potencies, found in close relationship to the small blood vessels. In the bone marrow, particularly in the zone of erosion of cartilage, the perivascular connective tissue cells are reticular cells. Moreover, *endosteal cells,* associated with bone marrow as well as with bone tissue, are also reticular cells. *Periosteal cells* form the deeper layer of the periosteum, sometimes called the *cambium layer;* unless participating in osteogenesis and recognizable as osteoblasts, they may be identified only by their location.

Criteria of Origin. All types of connective tissue derive from embryonic mesenchyme. Some *mesenchymal cells* persist in an undifferentiated form in the adult organism, with the capacity of differentiating into new cell types, e.g., osteoblasts. Connective tissue cells may arise in inflammatory processes, such as the reaction to transplants and to injury. The cells may be of local *histogenic* origin, arising from histiocytes (macrophages) or from lymphoid wandering cells; or *hematogenic,* arising from monocytes and, according to some, also from lymphocytes. Connective tissue cells of inflammatory origin may differentiate into fibroblasts; it is still uncertain whether they may be transformed into the cells of bone.

Criteria of Function. The term *fibroblast* implies that the cell produces connective tissue fibers. Some histologists prefer to reserve the term for cells which have differentiated irreversibly and which, by definition, are not capable of transformation into the cells of bone. Others describe the transformation of fibroblasts into bone cells but not into other connective tissue cells. The issue is frequently avoided by reference to spindle-shaped or fibroblast-like cells. *Reticular cells* are so named because of their intimate relationship with reticular fibers, which they are active in forming. Like the mesenchymal cells of the embryo, they may turn into all types of blood and connective tissue cells; reticular cells of the bone marrow also have osteogenetic potencies.

Criteria of Potencies. A cell with the capacity to transform into an osteoblast and to form bone has *osteogenetic potency.* Such a potency is latent; when it manifests itself, either spontaneously or as a result of experimental intervention, the cell exhibits *osteogenetic activity.* A reversible change in cellular activity is *modulation;* an irreversible change in potency is either a *differentiation* or a *dedifferentiation.* In many instances it is not possible to ascribe specific potencies to cells without having observed the corresponding activities. Connective tissue cells with the capacity to undergo differentiation or modulation and to exhibit osteogenetic or other specific activities are called *mesenchymal cells* in embryonic life. In postfetal life their nature is not always clear; some *perivascular connective tissue cells* have these potencies, as do the reticular cells in the bone marrow.

TERMINOLOGY OF CONNECTIVE TISSUE CELLS

There are many areas of uncertainty concerning the cells of connective tissue. Without attempting to resolve these uncertainties, we shall use the following terms for designation of connective tissue cells, with the meanings as indicated.

Mesenchymal Cell. An embryonic connective tissue cell, with an outstanding capacity for proliferation and capable of further differentiation, as into reticular cells or osteoblasts, is a *mesenchymal cell.* When persisting in the adult organism, these cells are usually arranged in loose connective tissue along the small blood vessels and as reticular cells in relation to reticular fibers. They are identified by location as well as by the capacity to differentiate into other cell types, such as into smooth muscle in the formation of new arteries in inflammatory processes; into phagocytes; and into bone.

Reticular Cell. The cell of reticular connective tissue, including the stroma of the bone marrow, where it retains both osteogenetic and hemopoietic potencies, is called a *reticular cell.* It is identified by its location, morphology, potency, and direct origin from mesenchymal cells.

Endosteal Cell. The endosteum is a condensation of the stroma

of the bone marrow; its cells are reticular cells, identifiable as *endosteal cells* by their location.

Fibroblast. This is a spindle-shaped cell of the loose and dense connective tissue, with the capacity to form the fibers of these tissues. Opinion differs as to its ability to form bone; we shall not refer to this cell as a precursor of bone cells.

Periosteal Cell. The outer layer of the periosteum is a network of dense connective tissue fibers and fibroblasts. Osteogenetic potencies have not been demonstrated for this layer. The inner layer, sometimes called the *cambium layer*, is actively osteogenetic during growth; in adult life its cells are identified only by their location and by activation of their osteogenetic potencies after an injury.

Connective Tissue Cell. By far the greater number of cells of connective tissue are fibroblasts; these are of doubtful potency with respect to the formation of bone. Where we wish to imply that cells of connective tissue may still possess osteogenetic potency, we shall use the general, but noncommittal, term *connective tissue cell.* When desirable for purposes of clarity, we may further characterize these cells by such terms as *perivascular connective tissue cells, undifferentiated connective tissue cells,* or *young connective tissue cells.* At times we may also use the descriptive term *spindle-shaped cells.*

ORIGIN OF BONE

Under appropriate conditions, any of the foregoing cells, with the possible exception of fibroblasts, may assume the form of osteoblasts and play an active part in osteogenesis. In so doing, they form collagenous fibers and deposit the ground substance of bone. As the interstitial substance surrounds them, they become osteocytes. As resorption occurs, osteoclasts appear in numbers, arising either from osteoblasts or osteocytes or directly from the reticular cells of the bone marrow. At any stage in these transformations the process may be reversed; the cells characteristic of bone are then no longer recognizable. They may assume the forms of the connective tissue cells from which they are derived, while still retaining

their osteogenetic potencies. Figure 4 illustrates the relationships of connective tissue cells to bone.

In summary, bone arises in embryonic life by differentiation of mesenchymal cells into osteoblasts; some of the mesenchymal cells may first transform into reticular cells of the bone marrow before becoming osteoblasts. In postfetal life the growth and reconstruction of bone occur mainly by osteogenetic activity on the part of

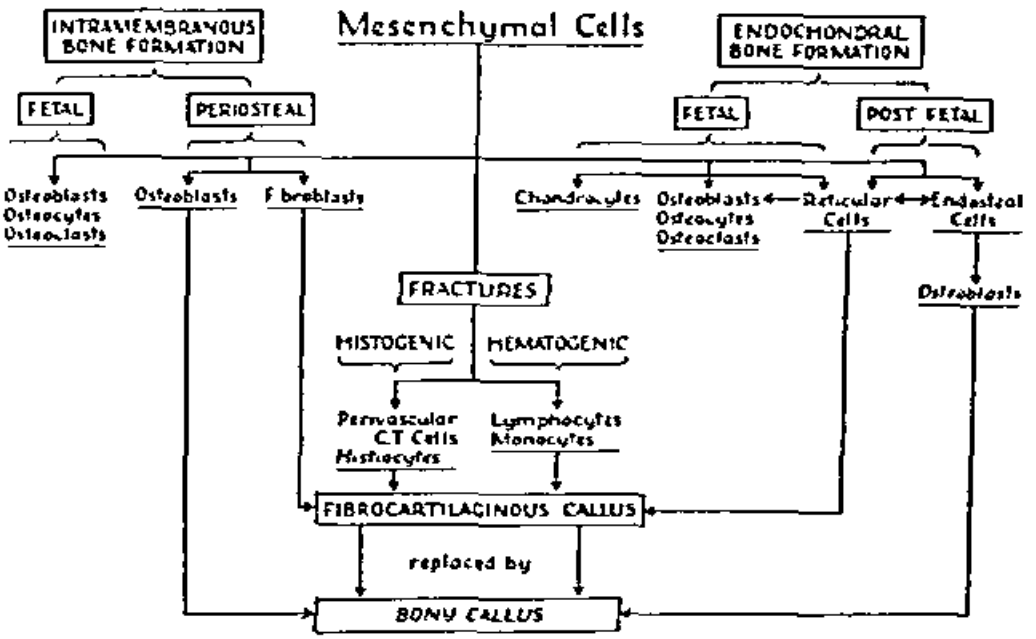


FIG 4.—Diagram to illustrate origins and transformations of the cells participating in the histogenesis of bone and in the healing of fractures. All these cells are derived from mesenchyme and progress through different pathways in different locations. The repair of fractures depends upon the mobilization of a variety of cells, each contributing to the formation of the provisional fibrocartilaginous callus or to bony union.

reticular cells, endosteal cells, and periosteal cells; all of these cells assume the form of osteoblasts while engaged in osteogenesis.

In later chapters the postfetal osteogenesis that occurs in the healing of fractures or following transplants of skeletal tissues will be examined. It will be shown that, when bone is transplanted to a soft tissue, it may lead, by induction, to formation of bone by the connective tissue cells of the host, under the influence of the transplant. Under these conditions the cells that may engage in osteogenesis are the undifferentiated connective tissue cells of the

host. In addition, histogenic and hematogenic cells of inflammatory origin may possibly participate in osteogenesis; whether this is the case is not proved. In the healing of a fracture the same reservoirs of connective tissue cells are available; to them are to be added the endosteal and periosteal cells in the fracture area. They are the first to form bone.

FORMATION OF BONE

The formation of bone, particularly in the embryo, may be preceded by the laying-down of a cartilage model; or it may occur by direct transformation of connective tissue. When bone is formed in such a manner as to replace cartilage, either in embryonic or in postfetal life, the process is *endochondral* or *intracartilaginous ossification;* when the transformation is direct, without the presence of cartilage, this is *intramembranous ossification.* It is convenient to describe these two forms of ossification separately.

Bone is characterized by deposition of the bone mineral within an organic matrix. This proceeds, during development and growth, both in the matrix of the cartilage model in advance of bone formation, and in the bone matrix as it is laid down. Calcification will accordingly be described with the histogenesis of bone.

INTRAMEMBRANOUS OSSIFICATION

Intramembranous formation of bone occurs in every part of the body—in the shafts of the long bones and in the continuous growth, internal reconstruction, and remodeling of every bone. In most vertebrates it is the only form of growth or reconstruction of bone that continues throughout life, after growth in length of the long bones has ceased.

Intramembranous ossification, in its very earliest stages and in its least complicated form, is best seen in the formation of the embryonic calvarium. Here, in the places where bone is about to appear, the intercellular substance between the cells of the connective tissue, previously indistinguishable from other connective tissue surrounding it, increases in amount and density. It assumes a more homogeneous appearance and becomes eosinophilic. Simultaneously, the connective tissue cells increase in size and

assume the form of osteoblasts. At about the same time, beginning calcification in the matrix is first demonstrable with the von Kóssa stain. It is seen as scattered granules of silver, associated with the interstitial tissue. The content of bone salt of the newly formed matrix rapidly increases, as the transformation to bone becomes complete.

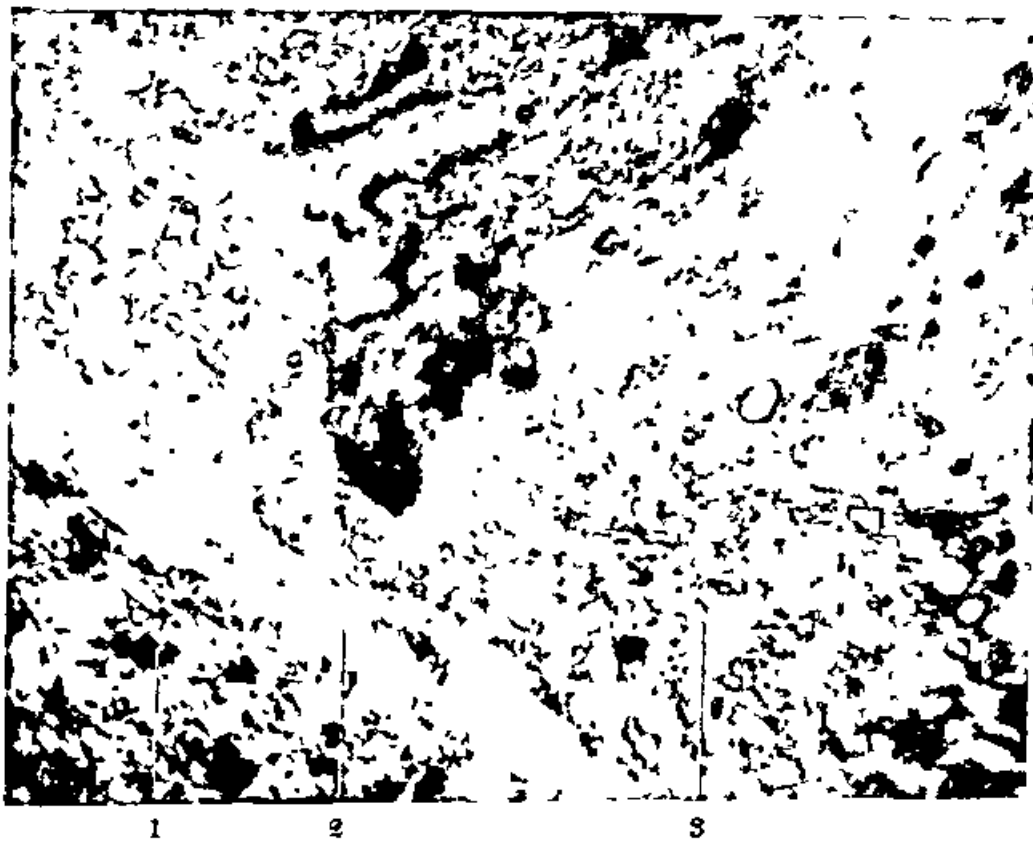


FIG 5 —Electron micrograph of undecalcified tibia of a rat, fixed with osmic acid *1*, Zone of calcification; *2*, preosseous tissue; *3*, cross-sections of fibers. (From original of Fig 9, Knese and Knoop, Zeitschr. f. Zellforschung, 48:465 Reproduced by courtesy of the publishers)

Pommer introduced the concept of *physiological osteoid*, as a necessary stage in the formation of bone by apposition. According to this concept, the first step is deposition of a non-calcified preosseous or osteoid tissue; calcification then follows, and bone is the result. McLean and Bloom, utilizing sections of undecalcified bone, stained with silver nitrate to demonstrate the bone mineral, and observed with the light microscope, concluded that the matrix may

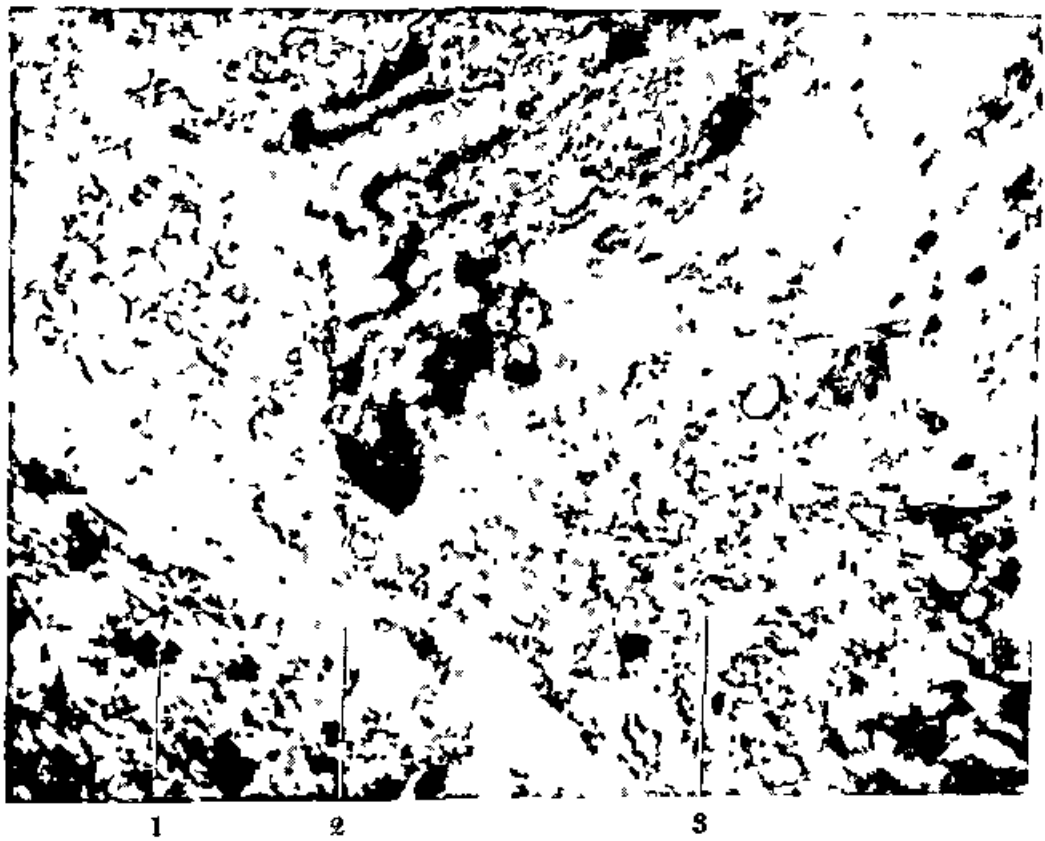
be regarded as calcifiable as soon as the tissue is recognizable as bone, and that the delay sometimes observed in the deposit of mineral may be ascribed to a lag in the supply of the necessary materials. Frost *et al.* have studied the layers of unmineralized matrix from 5 to 30 microns thick, usually applied on a pre-existing bone surface and designated as *osteoid seams*. They found a normal incidence of such seams, in human material, in terms of the longitudinal vascular channels in diaphyseal cortex, as follows: at age 5, about 10 per cent; at age 15, 3 per cent; at age 30, 0.8 per cent; and at age 70, 0.8 per cent. There were variations from one bone to another and, in general, there were higher values in the axial skeleton than in the appendicular skeleton. Observations with the electron microscope appear to demonstrate that there is always a thin layer, 1 micron or less, of uncalcified preosseous tissue during the formation of bone, even in animals with an optimum intake of minerals. Whether this requires a return to the concept of physiological osteoid, in the sense of Pommer, is a matter of opinion; certainly, the broad osteoid borders observed by him were indicative of a minimal rachitic state, rather than of physiologic bone formation (Fig. 5).

INTRACARTILAGINOUS OSSIFICATION

Cartilage models of most of the bones of the skeleton are formed during embryonic life. Initially, these models are made up of hyaline cartilage, relatively free from glycogen, from phosphatase, and from the enzyme systems concerned in glycolysis. At a certain stage of embryonic life, characteristic of each bone in each species, the hyaline cartilage undergoes changes usually described as degenerative, and the cells enlarge. At this time, the hypertrophic cartilage cells accumulate glycogen, and the glycolytic enzymes and phosphatase appear.

The changes in the cartilage cells occur in certain locations, destined to become the ossification centers for the shafts of the bones. As the cells undergo these changes, there is an ingrowth from the periosteum, in one or more places, of vascular mesenchyme. This penetrates the areas of hypertrophic cartilage cells and replaces them with primitive bone marrow, leaving only

assume the form of osteoblasts. At about the same time, beginning calcification in the matrix is first demonstrable with the von Kóssa stain. It is seen as scattered granules of silver, associated with the interstitial tissue. The content of bone salt of the newly formed matrix rapidly increases, as the transformation to bone becomes complete.



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scattered, short fragments of matrix, upon which osseous tissue is deposited. The entire process of ossification in cartilage models begins in a relatively small number of foci, originating from the mesenchyme forming the perichondrium or periosteum. The mesenchymal cells have osteogenetic and hemopoietic potencies, and from them are formed the ossification centers of the bone and the primitive bone marrow.

From the ossification centers, replacement of cartilage by bone and primitive bone marrow extends centrifugally, until the marrow cavity is free of cartilage cells. Only fragments of cartilage matrix are left; some of the cartilage cells may survive and become osteoblasts. It has been customary to describe this replacement as invasion and erosion, as though the force behind it lies in the mesenchyme, driving it to destroy the cartilage. It is at least equally reasonable to assume that the sequence of events begins with the changes in the cartilage cells themselves and that the ingrowth of the mesenchyme is a response to these changes. In any case, the widespread replacement of cartilage is slowed down only as it approaches the portion of the model destined to form the epiphysis and the epiphyseal cartilage. Here, the line of further growth of bone is established, and the replacement of cartilage continues in a more orderly fashion. Growth in length of the long bones is thus a direct continuation of the intracartilaginous ossification within the embryonic cartilage model.

At the time that foci of hypertrophic cells are seen in the cartilage models of bone, or shortly thereafter, calcification of the cartilage matrix is demonstrable. In certain bones of the embryonic rat this calcification may begin before penetration by mesenchyme, so that the ingrowth from the periosteum is into an area in which calcification has already taken place. In the bones of larger animals calcification may not occur until penetration is under way, but it proceeds in advance of the invading tissue for at least the space of a few hypertrophied cells. In both cases, the effect is that replacement of the embryonic cartilage model by bone is guided, just as it is later in the growth of bone, by a network of calcified cartilage matrix (Fig. 6).

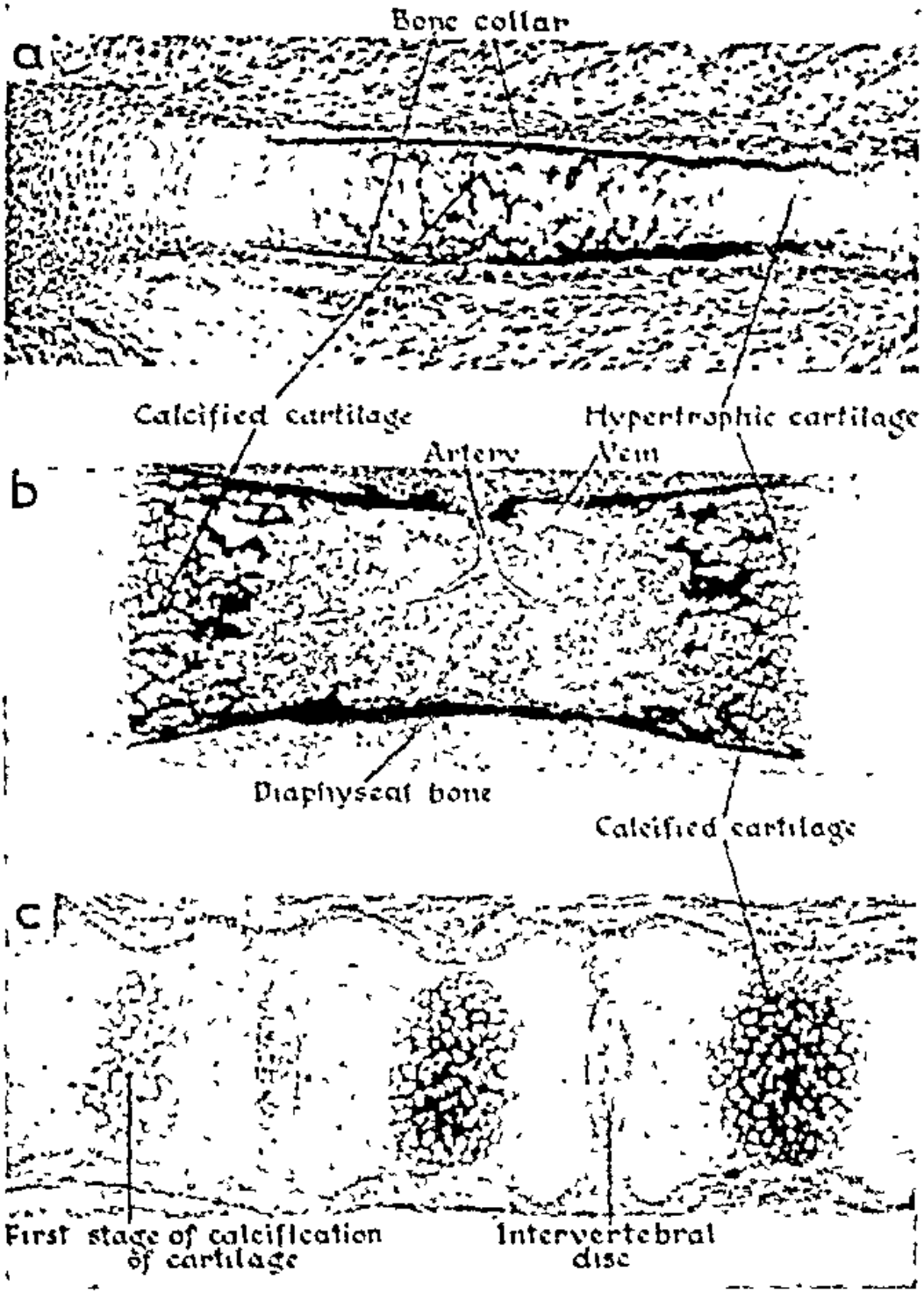

Fig. 6.—Sections to illustrate calcification of bones developing from cartilage models in embryonic and newborn rats. (a) Longitudinal section through second rib of 18-day rat embryo. The calcification of the periosteal bone collar is further advanced than that of the cartilage. ×101. (b) Section of metatarsal of 4-day rat. The matrix of the hypertrophic cartilage is not completely calcified. ×50. (c) Section through cartilage models of bodies of three vertebrae of 20-day rat embryo, showing three stages in the calcification of the model. Silver nitrate–hematoxylin-eosin. ×50. (From original of Pl. 5, Figs. 8, 9, 10, Bloom and Bloom, Anat. Rec., 78:523. Reproduced by courtesy of the publishers.)

Also, at about the same time the cartilage model is being penetrated, a thin layer of calcified subperiosteal bone is formed, encircling the shaft of the model and constituting the *periosteal collar.* Intramembranous formation of bone in this location continues by osteoblastic apposition, and calcification of the osseous tissue follows.

After the line of growth is established and at a time characteristic for each species and each bone, the cartilage forming the epiphyseal ends of the bone is invaded, again by ingrowth of mesenchyme, and replacement of the cartilage by bone follows. The epiphyseal cartilage disk remains oriented toward the marrow cavity of the shaft, its epiphyseal surface commonly being covered by a plate of bone. There develops within the epiphysis a framework of spongy bone. This gives strength to the ends of the bone and forms the support of the articular cartilage, which is also supported by plates of underlying bone.

ENDOCHONDRAL GROWTH OF BONE

The epiphyseal cartilage is a portion of the embryonic cartilage model that persists through adolescence, proliferates, and participates in the growth in length of the long bones. This growth occurs by a continuous ingrowth of capillaries into the proliferating epiphyseal cartilage, accompanied by mesenchymal cells with osteogenetic potencies; the invaded cartilage is thus replaced by diaphyseal bone. The net result is that the cartilage disk remains at an approximately constant thickness, being replaced on the diaphyseal front, while new cartilage cells, arranged in rows, arise from the epiphyseal face. The diaphysis increases its length by the amount by which it replaces cartilage. Growth, then, occurs in two places: (1) in cartilage by division of cells; and (2) in diaphyseal bone by replacement of cartilage. New bone is laid down on a framework of calcified cartilage matrix, which persists when the cartilage is invaded (Fig. 7).

GROWTH APPARATUS

The process by which the long bones continue to grow in length through adolescence is similar to that of the replacement of em-

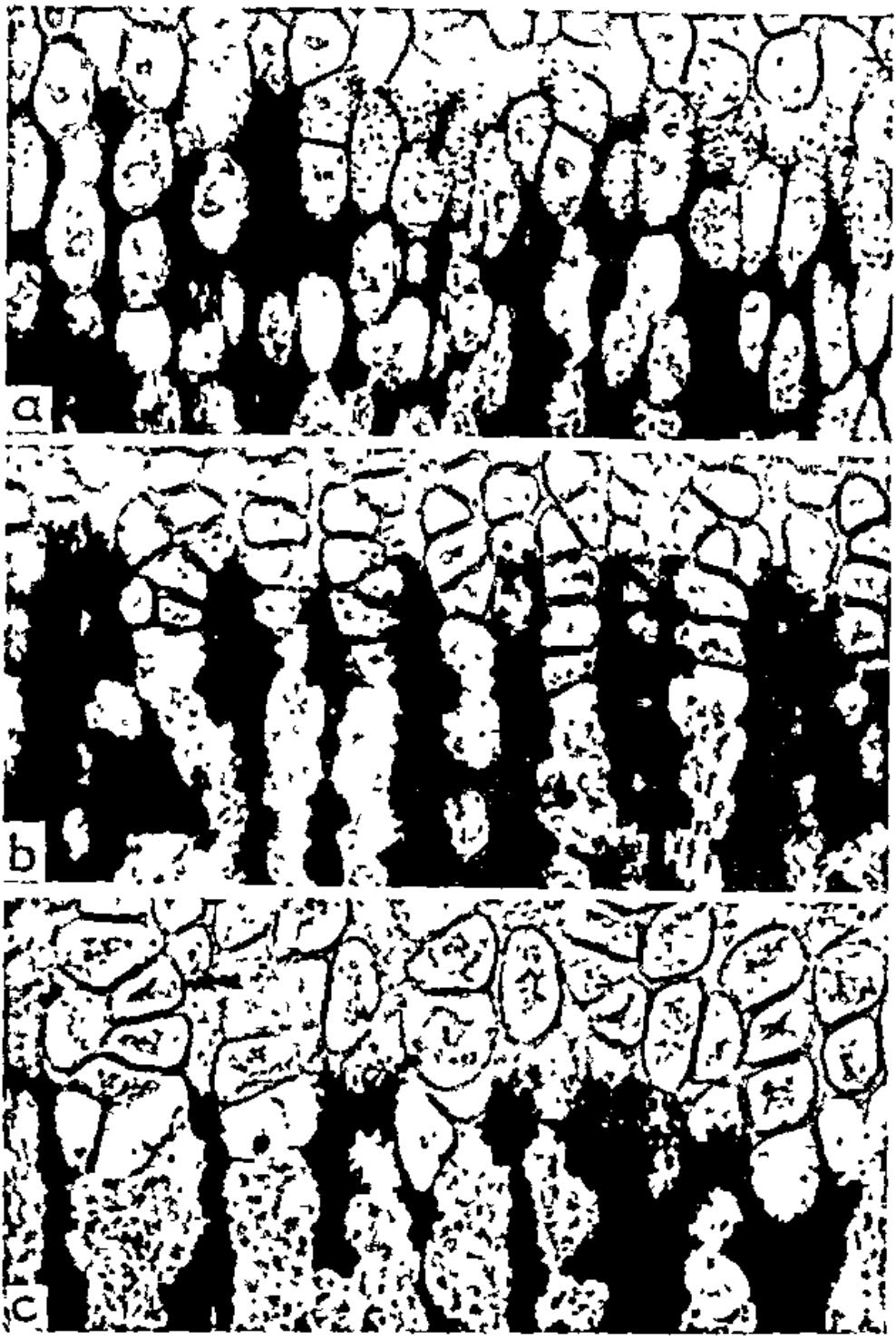

FIG. 7.—Sections through hypertrophic cartilage and zones of provisional calcification in epiphyseal cartilages, to illustrate progress of calcification in the growth in length of the long bones. (*a*) Proximal end of tibia of normal rat, age 28 days; (*b*) distal end of radius of normal puppy, age 34 days; (*c*) costochondral junction, same puppy. Note interdigitation of calcified cartilage matrix and cartilage in *a* and *b*, absence of interdigitation in *c*; calcified cross-partitions in *a*; absence of osteoid in primary spongiosa in all. Silver nitrate–hematoxylin-eosin ×245. (From original of Pl. 2, Figs. 2, 3, 4, McLean and Bloom, Anat. Rec., 78:357. Reproduced by courtesy of the publishers.)

bryonic cartilage models by bone, with the exception that the epiphyseal cartilage disk and its surrounding structures become organized and oriented to perform a specific function. This requires a complex mechanism, which we have termed the *growth apparatus;* unless this mechanism functions as a whole, growth in length cannot occur or is distorted.

The growth apparatus is based on the epiphyseal cartilage but is not limited to this structure. The epiphyseal face of the disk is made up of cartilage matrix, within which cartilage cells are imbedded. These continue to divide, giving rise to new cells, commonly arranged in rows; for this reason the dividing cells are known as *mother-cells,* or *row mother-cells;* the rows are separated from one another by cartilage matrix. Replacement of the cartilage cells by bone occurs at the opposite face of the cartilage, i.e., the diaphyseal face or front.

Viewed in sagittal section, the cartilage cells become larger as they near the diaphyseal front and develop vacuoles in their cytoplasm. The nuclei swell and lose most of their chromatin, and the cells degenerate; at this stage they are known as *vesicular* or *hypertrophic* cartilage cells and are ready for penetration by the vascular connective tissue. The *primary spongiosa* is a direct and unreconstructed continuation of the cartilage matrix, the hypertrophic cartilage cells being replaced by vascular connective tissue. Osteoblasts are numerous at the junction of the spongiosa with the cartilage, but, as a rule, little or no new bone is deposited on the matrix of the primary spongiosa.

If the epiphyseal cartilage and the primary spongiosa are viewed in serial cross-sections, they resemble a honeycomb in structure, each compartment of which contains a column of cartilage cells, growing out from the mother-cells and undergoing hypertrophy as they approach the cartilage front. Into each of these columns there grow from the bone marrow one or more minute blood vessels, accompanied by perivascular connective tissue cells. The blood vessels penetrate the hypertrophied cartilage cells, most of which disappear and are replaced by vascular ingrowth.

In advance of the invading blood vessels, usually to a depth of two to four cells in the cartilage, the matrix making up the walls of the columns becomes calcified. This forms the *zone of provisional calcification*, virtually indentical with the *zone of growth*, within which the cartilage cells are displaced by the tissue advancing from the bone marrow. This calcification serves a double purpose; it guides the blood vessels in their progress into the columns of cartilage cells, and it affords structural strength, by providing interlocking or interdigitation between the bony diaphysis and the epiphyseal cartilage, bridging the zone of growth. Calcifiability of the cartilage matrix in the region of the hypertrophic cartilage cells is believed to be conferred upon the matrix by some activity of the adjoining cells.

The normal functioning of the growth apparatus has been subjected to mechanical interference in experiments reported by Trueta and Amato. If a plastic membrane is placed in the center of the epiphysis to isolate the marrow from the epiphyseal plate, normal division and row formation of cartilage cells does not occur, owing to the interposition of a barrier to the nourishment of the row mother-cells by the blood vessels of the epiphysis. If the membrane is interposed between the metaphysis and the epiphyseal plate, the columns of cartilage cells persist; however, calcification is absent or delayed, and the chondrocytes persist. Here, the barrier interferes with the ingrowth of blood vessels from the metaphysis into the growth apparatus.

The barrier that prevents vascular invasion of the columns of cells in the epiphyseal cartilage has a pathologic counterpart in a condition arising from faulty growth of cartilage, usually hereditary and known as dyschondroplasia or Ollier's disease; the growth apparatus fails to function, and the normal sequence of cartilage degeneration, calcification, and growth is interrupted. A comparable failure of the growth apparatus may be induced by chemical or metabolic interference. If rats are fed a diet deficient in vitamin D and phosphorus, and *rickets* is produced, the hypertrophic cartilage cells persist and accumulate, and the growth sequence fails.

SECONDARY SPONGIOSA

The *secondary spongiosa is a vaulted structure*, resting upon the primary spongiosa and transferring the stresses to the shaft. It is a direct continuation of the primary spongiosa, subjected to much more thinning-out and reconstruction, and it is in the secondary spongiosa that deposition of new bone on the cores of calcified cartilage matrix is most prominent. Such bone is ordinarily calcified as it is laid down, with only a thin layer of uncalcified osseous tissue demonstrable by the electron microscope; in well-nourished animals, borders of uncalcified osteoid tissue, visible under the light microscope, are not common. Most of the calcified cartilage matrix disappears during growth and reconstruction. Although cartilage cells at times transform directly into bone cells, this is quantitatively an unimportant part of bone formation or growth in the higher animals. Nothing comparable to the growth apparatus of endochondral ossification is apparent in the growth of bone formed by intramembranous ossification.

ORGANIZATION OF BONE

Bones are organized on two levels: (1) as a tissue, and (2) as organs. Bone as a tissue has a more complex pattern than is apparent in either loose or dense connective tissue. Bones as organs are highly specialized in relation to embryonic development, to growth, to function, and to regeneration following injury.

The organization of bone as a tissue is displayed in the structure of the organic matrix, and in the relation of this matrix to the osteocytes, with their lacunae and interconnecting canalicules. The fibers of compact bone, instead of being arranged in a random fashion, are oriented to the structure of the haversian systems.

In primary ossification, beginning in embryonic life, the fibers of bone are interwoven, without a definite internal structure. Subsequently, and early in the postfetal life of man, tunnels are hollowed out in the osseous tissue, by resorption from the marrow cavity, and these tunnels are known as primitive haversian systems. After the age of one year, all new bone formed in the diaphyses of the long bones is laid down in layers, or lamellae.

The unit of structure of compact bone is the *haversian system*, or *osteon*. This is, when fully formed, an irregularly cylindrical and branching structure, with thick walls and a narrow lumen—the haversian canal. The canal carries one or more blood vessels, mainly capillaries and venules. The cylindrical osteons are usually oriented in the long axes of the bones. The walls have a definite

FIG. 8.—Sections through compact human bone, to illustrate haversian systems *Left*, cross-section, ×180; *right*, longitudinal section, ×215 (From Weinmann and Sicher, *Bone and bones* [St Louis: C. V. Mosby Co., 1947], Figs. 11 and 13, pp 30 and 32. *Reproduced by courtesy of the publishers*.)

lamellar structure, the fibrils of each lamella running spirally to the axis of the canal; the direction of the fibrils changes from layer to layer of the successive lamellae. The haversian system, in addition to being arranged around a central canal, includes large numbers of lacunae, housing the osteocytes and interconnected with one another and with the lumen of the canal by means of branched canalicules (Fig. 8).

Bone, either intramembranous or intracartilaginous, is first

formed as trabeculae of spongy bone, with irregular communicating cavities, filled with bone marrow, and with centrally located blood vessels. As the *osteoblasts covering the bone* produce layer after layer of new lamellae by apposition on the bony surfaces, the marrow spaces are reduced to such an extent that only a small canal remains about a blood vessel. This is, then, a *primitive* or *primary haversian system*, or osteon, with its haversian canal, and it represents the initial state of *compact bone*. In certain small animals, such as the mouse and rat, organization of bone does not progress beyond this stage. In larger animals, however, *secondary osteons* are produced by formation of cylindrical *absorption cavities* or tunnels, which are then filled in by deposition of concentric layers or lamellae of bone, again leaving, in each case, a haversian canal. Successive generations of haversian systems are then formed throughout the life of the animal. They cut across the lines of the earlier osteons, leaving irregularly shaped areas of *interstitial lamellae*. Additional *circumferential lamellae* are formed under the periosteum and endosteum. Compact bone thus consists of lamellar bone, arranged in a variety of ways: in relatively new and regularly shaped osteons; in portions of older osteons, remaining as interstitial lamellae; and in circumferential layers near the surfaces of the structure. All of these lamellae are alike in structure and function; their differences are incidental to the sequences occurring in their formation.

EMBRYONIC INDUCTION OF BONE FORMATION

The organization of the histogenesis of bone and of the development and growth of the bones has been made the subject of a detailed study by Lacroix, and the reader is referred to his monograph and his subsequent publications. He has developed the theory of organization of bones by reference to two concepts, both familiar to students of experimental embryology. The first is that of *induction*, which means that a tissue influences the development and differentiation of cells near by, to the end that they exhibit potencies not previously in evidence. The second is that of an *organizer*, or organizers, by which is understood one or more chemical sub-

stances that can influence decisively the direction that further development of a tissue may take.

The first example of induction given by Lacroix is that the exclusively cartilaginous part of the growth apparatus is capable, during proliferation, of exciting formation around it of a *perichondrial ring* of the ossification groove. His conclusion is that the existence of such a mechanism implies the intervention of an *organizing substance*. As a second example of induction, he describes experiments in which hyaline cartilage, normally not osteogenetic, is transplanted and kept in contact with an epiphyseal cartilage. Under these conditions it acquires the structure and properties of the epiphyseal cartilage. He calls this "*assimilatory induction of* endochondral ossification." The inducing tissue imposes its own organization on the graft, and the occurrence of assimilatory induction creates a strong presumption in favor of the existence of an organizer, which he has named "*osteogenin*."

The thesis that induction occurs from an extractable diffusible substance rests mainly on the occurrence of osteogenesis following the injections of suspensions or solutions, obtained by extraction of bone substance, into the muscle of rabbits; this phenomenon is still a subject of some controversy, and is given further consideration in chapter xiii. As a general rule, in order to induce the cells of a host tissue to undergo a transformation, enabling them to form a new and different tissue, prolonged contact with the inductor is necessary; this condition is not met by a single injection of a diffusible substance into the muscle of a host. The formation of cartilage, subsequently undergoing transformation into bone, has been demonstrated by Bridges and Pritchard, following implantation of small pieces of devitalized muscle under the kidney capsule. This suggests that a similar mechanism may be responsible for formation of bone when muscle is devitalized *in situ* by injections of alcohol, with or without substances extracted from bone.

INTERNAL RECONSTRUCTION OF BONE

Bones increase in length by the functioning of the growth apparatus and in diameter by apposition of new periosteal bone,

while the marrow cavity is being enlarged by resorption at the endosteal surface. In addition to the changes in size and shape of the bones, as a result of the remodeling incident to growth, there is continuous internal remodeling throughout the life of the individual; this serves an important physiologic function, essential to homeostatic control of the calcium of the blood, and, therefore, to life itself.

In compact bone there is first the formation of absorption cavities, described by Tomes and De Morgan in 1853. The appearance of these cavities is associated with the presence of osteoclasts, and the cavities are extended, generally in the long axis of the bone, until they assume the form of tunnels Regulation of the tunnelling process is not understood; it appears to be independent of parathyroid activity, usually associated with osteoclastic resorption in other locations.

The tunnel, or absorption cavity, as seen in sections, contains blood vessels and connective tissue cells. While resorption is in progress, the cavity is lined with osteoclasts; when resorption comes to an end and rebuilding begins, these are replaced by osteoblasts. The tunnel is then filled in, from its walls toward the center, by apposition of bone in successive concentric layers or lamellae, incorporating the osteocytes, their lacunae, and the canalicules. Wherever the central blood vessel branches, a lateral branch of the osteon is formed to surround it; some of these become the canals of Volkmann and communicate with the marrow cavity or the periosteal surface of the bone. The formation of new layers continues until the canal reaches its final diameter, usually approximately 20 microns.

Using lead as a marker, Vincent has studied the rate of formation and of maturation of new osteons. An average absorption cavity in a dog takes roughly three weeks to form, this being the period required for tunnelling or excavation. The building of the new osteon, including partial mineralization of the organic matrix, requires some six to twelve weeks. Primary mineralization of the matrix, to about 70 per cent of the final content, occurs rapidly during and immediately after the deposition of new layers of organic material;

completion of secondary mineralization, to maximum density, takes much longer and has been found to be incomplete for as long as eighteen weeks.

Frost *et al.*, using tetracycline as a marker, found the mean osteon formation time in a fifty-seven-year-old man to be five weeks. The biologic half-life of the osteons was calculated as 2.7 years for the femur and 8.6 years for the tibia. The percentage of

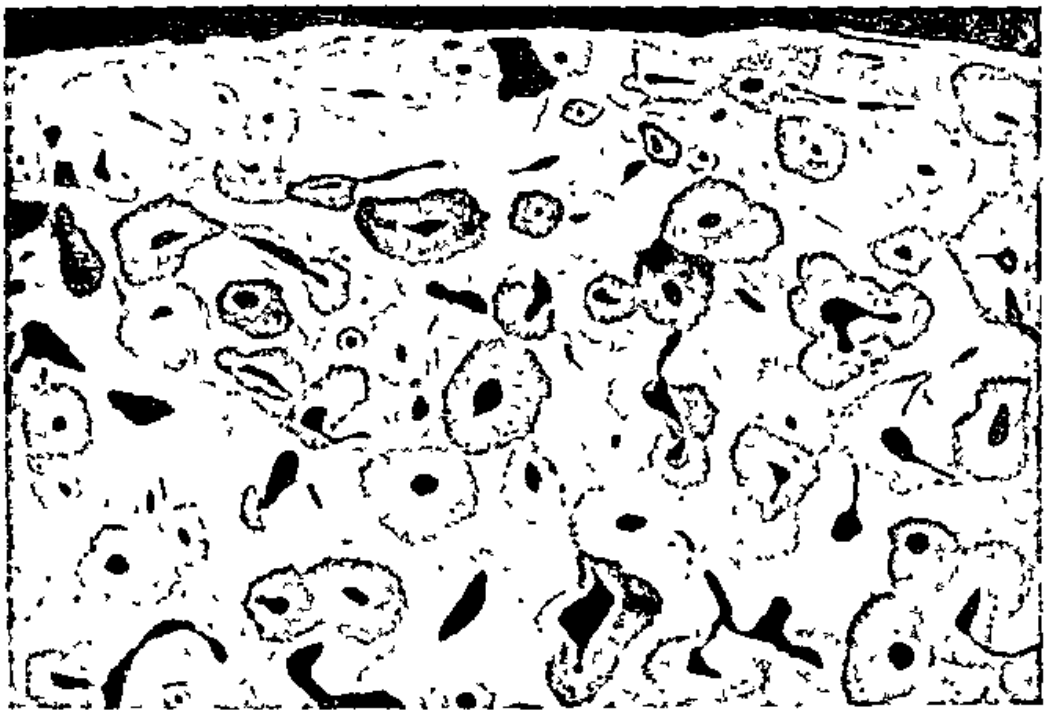

FIG 9.—*Microradiograph of normal human compact bone, from cross-section of fresh tibia of a woman, age 47. Section, 100 μ thick, cut with high-speed rotary saw without embedding and without further treatment. Radiograph made with 9 KV X rays, on Eastman Kodak 649-0 spectroscopic plate. ×30.* (Reproduced by courtesy of R. E. Rowland, Argonne National Laboratory.)

the mass of bone turned over, i.e., resorbed and redeposited, per day, was 0.036 for the femur and fibula and 0.012 for the tibia. In another study, also with tetracycline, the same authors report that an average of 0.9 microns of new osteoid per day is formed in active haversian systems in adults.

ULTRASTRUCTURE OF BONE

While the intimate structure of bone, as observed with the light microscope, has been well described for many years, the exploita-

tion of new biophysical techniques has added information concerning the details of fine structure and has increased knowledge of the relation of structure to function; special emphasis on the interrelations of the chief components of bone is necessary for an understanding of its functions.

The terms *fine structure* and *ultrastructure*, as well as *submicroscopic*, have been used to indicate particles or structures not resolvable by the light microscope. High-resolution electron microscopy refers specifically to dimensions of 30 A or less. At the electron microscope level, information has been obtained concerning the fibrillar structure of bone and the characteristics of collagen fibers, as well as about the form of the crystals of the bone mineral and their relationship to the collagen fibers and to the ground substance. A beginning has been made on the ultrastructure of ground substance, until recently described as amorphous. The potentialities of the electron microscope for elucidating the fine structure of bone, thus permitting further insight into its functions, have by no means been exhausted.

Electron microscopy of bone has been aided by X-ray diffraction, both high-angle and low-angle. High-angle diffraction, particularly with microdiffraction techniques, has been of assistance in studying the crystallographic properties of bone tissue. Low-angle diffraction has given information about the dimensions of the particles and their orientation.

Microradiography requires the passing of X rays through thin sections and the recording of differences in absorption of the radiation on photographic plates or film, thus permitting enlargement of the image. Whereas ordinary histologic sections of bone do not reveal differences in the density of individual osteons, such differences are clearly demonstrated in microradiograms; the younger osteons are less mineralized and, consequently, less dense than the older ones. This has focused attention on the mineralization of osteons, and it is readily demonstrable that the new and less dense osteons account for most of the uptake of radioactive calcium, strontium, and phosphorus (Fig. 9).

The results obtained on normal bone tissue by microradiography

have been confirmed by microinterferometry. In addition, microinterferometric measurements made on decalcified tissue have shown that the content of organic material varies little from one osteon to another, indicating that the differences seen in microradiograms depend solely upon the content of mineral. The polarizing microscope reveals the arrangement of the molecules in calcified tissue; the close relationship between collagen and the mineral phase of bone was observed by this means as early as 1923.

The numbers of *mitochondria* in periosteal osteoblasts have been found to increase up to the period of maximum rate of bone formation, and to fall sharply thereafter. The presence of a *sex chromatin body* similar to that reported in other tissues has been demonstrated in the nuclei of osteoblasts, osteocytes, and periosteal cells from female dogs and cats.

RELATION OF STRUCTURE TO FUNCTION

When a radioisotope—for example, strontium-90—has been administered to an animal and undecalcified sections of compact bone have been made, it is possible to prepare photomicrographs, microradiograms, and autoradiograms, all of the same section. These may be enlarged to the same size and compared (Frontispiece). The radioisotope is not only taken up chiefly by the newer and less dense osteons; by far the greater portion of it is taken up by a thin layer of new and only slightly mineralized matrix, which may not be seen at all in microradiograms, but may be visible in the section under the light microscope. When this layer is absent, no appreciable amount of radioisotope may be taken up by a particular osteon, even though the osteon as a whole is incompletely mineralized and, hence, of a density comparable to that of others which do take up the isotope. These combined techniques afford a record of the sequence of events in the growth and mineralization of bone, not seen by any one of the techniques alone.

Such findings as these have given strength to the view, to be treated at length later in this volume, that there is a labile fraction of the bone mineral, located chiefly, if not exclusively, in the newly formed and incompletely mineralized osteons, and more partic-

ularly in the newest layers lining these osteons. This labile fraction of mineral, associated with bone of maximum reactivity, accounts for the rapid transfer of mineral, in both directions, between bone and the fluids of the body. When the blood plasma is depleted of calcium, there is a rapid movement of this element from the intercellular fluids, which serve to buffer any rapid changes in the Ca^{++} content of the plasma; the calcium thus transferred from the intercellular fluids to the plasma is then replenished from the labile fraction of the bone mineral. As a rough guide, it may be said that the transfer of calcium from intercellular fluids to plasma provides for minute-to-minute adjustment; from the labile fraction of bone to the intercellular fluid for the hour-to-hour adjustment; and from the stable fraction of the bone to the blood for the day-to-day adjustment. Taken together, the separate elements of this mechanism provide for the continued life and health of the organism, which without it would be subject to violent fluctuations in the calcium ion concentration in the internal environment.

Since the maintenance of a continuous supply of reactive bone, housing the labile bone mineral essential to life, depends upon the continuity of internal remodeling of bone, this remodeling, formerly believed to be determined by weight-bearing and other forces acting externally on the skeleton as a whole, is now seen to have an important metabolic function. Vincent has referred to the reactive bone, located in the new osteons, as *metabolic bone* and has stated that, when the immature osteons reach maturity and lose their special reactivity, this marks the transformation of metabolic bone to *structural bone*. At any given time the amount of metabolic bone in the skeleton amounts to less than one per cent of the less active, structural bone.

CHAPTER IV

Structure and Chemical Composition of Bone Matrix

The organic fraction of compact bone makes up as much as 35 per cent of the dry, fat-free weight. Only a small part of this is contributed by the cells; the remainder, impregnated with the bone salt, is the bone matrix. This has been investigated intensively by histologic, chemical, and histochemical methods, and numerous electron microscope studies have been reported.

The organic matrix has two chief components. Of these the most prominent is fibrillar in nature and is chemically a collagen, identical with, or closely related to, the collagens found in other connective tissues. Between the fibers is found a ground substance, whose best-characterized component is a mucopolysaccharide identified as chondroitin sulfate. The fluid portion of the intercellular elements of the bone matrix is small in amount; but from the rapidity with which dissolved substances, such as dyes and radioactive isotopes, diffuse in the bones when introduced into the circulation, it is apparent that such fluid as is present is in close relationship with the ground substance. It is stated that free fluid may be entirely absent from considerable areas of compact bone, especially in the adult. This has an important bearing on the freedom of exchange of ions between the bone and the blood.

COLLAGEN

Bone collagen is the substance which yields glue or gelatin when boiled. It makes up 90–96 per cent of the dry, fat-free weight of the organic matter of bone. Most studies on collagen have been made on material from other sources, but enough information concerning collagen from bone is now available to make it clear that it

shares common properties with that from other forms of connective tissue.

Collagen is characterized by its appearance in fibrils, with double cross-banding at intervals averaging 640 A (Fig. 10). It also gives a characteristic X-ray diffraction pattern. Chemically it is characterized by a low content of aromatic amino acids and a high content of pyrrolidine amino acids and glycine and by its specific hydrolysis by the enzyme *collagenase*. Certain collagens, such as those from the tendons of the rat's tail and from the swim bladder of fish, are soluble in dilute acid and may be reprecipitated in fibrils,

FIG 10.—Electron micrograph, illustrating collagen fibers in a section of decalcified human femur. Shadowed with uranium at an angle of 5·1. The major doublet bands of the collagen are spaced by 560 A in this area. Incomplete intermediate cross-bands are found in a few places. Continuity of the doublet bands across several fibers at right angles to the fiber direction is observed. Osmium tetroxide fixation. (From original of Pl. 3, Fig. 4, Robinson and Watson, Anat. Rec., 114:399. Reproduced by courtesy of the publishers.)

with typical periodic cross-banding, by the addition of electrolytes to the solution. The individual fibrils, 0.3–0.5 μ in diameter, are often collected into small bundles 3–5 μ thick.

Collagen is readily digested by pepsin in acid solution but resists digestion by trypsin in alkaline solution. On hydrolysis, collagen or gelatin yields especially high proportions of glycine (25 gm/100 gm of protein), of proline (14.8 gm/100 gm of protein), and of hydroxyproline (14.5 gm/100 gm of protein). Hydroxylysine is found only in hydrolysates of collagen and of gelatin, and in these the content of this amino acid is only about 1 per cent. Alpha amino groups are absent from collagen; aspartic acid, glutamic acid, alanine, and threonine, as well as glycine, are found as terminal amino residues in various forms of gelatin. There is some evidence that the ϵ-amino groups of lysine may participate in the nucleation of crystal formation. Collagen is poor in some of the amino acids essential to protein metabolism. There are species differences in the physical and chemical properties of collagens. The life span of the individual fibers is in doubt. The physiologic turnover of collagen in tendons is extremely slow; the same is true for bone.

Collagen from bone and cartilage is indistinguishable from collagen from skin, tendon, and other connective tissues. The role of collagen in the nucleation of crystal formation, as exhibited by collagen reconstituted from solution, is considered in detail in a subsequent chapter.

GROUND SUBSTANCE

Broadly conceived, ground substance may be defined as the extracellular and interfibrillar component of all connective tissues. Formerly described as amorphous, it is now believed to have an organization and ultrastructure of its own, or at least to exist in more than one phase. The relation of tissue fluid to ground substance is poorly understood. A working concept is that the ground substance varies in consistency and density from that of an interstitial fluid, i.e., an ultrafiltrate of plasma, to that of the basement membrane, and that it is coextensive with both; they represent its most fluid and most condensed portions, respectively. Related to this is

The amounts of mucopolysaccharides in bone are small; analytic data are scanty. Dry, fat-free human compact bone has been reported to contain 0.1–0.25 per cent of hexosamine (estimated as glucosamine hydrochloride), and 2.0–4.0 per cent of total reducing substance (estimated as glucose). Ox shaft bone has been reported to contain 0.3–0.4 per cent of a sulfated polysaccharide, composed of about equimolar concentrations of hexosamine, uronic acid, and sulfate and is a chondroitin sulfate; of the total content, only about 10 per cent was isolated. Eastoe and Eastoe reported the isolation of a mucopolysaccharide-protein complex obtained by lime-water extraction of air-dried bone powder (0.24 per cent by weight). On hydrolysis, they demonstrated both galactosamine, 7.67 per cent, and glucosamine, 1.23 per cent, together with 1.63 per cent sulfate-S. On paper chromatography, they found galactose, mannose, and xylose.

The results from analyses of bone are consistent with the finding of approximately 0.5 per cent of chondroitin sulfate in dentin. Another approach to the estimation of chondroitin sulfate in cartilage and bone is by analysis for total sulfate. The sulfate content of the organic matter of bone is about 15 per cent of that of cartilage. A similar contrast between cartilage and bone is revealed by autoradiographs following administration of S^{35} to suckling rats. The sulfated mucopolysaccharides of bone have been shown to exist in protein complexes. The highly polymerized molecules of chondroitin sulfate do not ordinarily move with the tissue fluid into the blood plasma.

Based upon determinations of hydroxyproline, as an index of the concentration of collagen, and total hexosamine, as an index of the concentration of mucopolysaccharide, it is believed that the ratio of mucoprotein to fibrous protein increases with age. Associated with these, the ratio of glucosamine to galactosamine also decreases in aging connective tissues; cartilage contains more keratosulfate with aging.

The plant proteases—papain, bromelin, and ficin—have been useful for the study of ground substance. When crude or inactivated papain is injected into a young rabbit the ear cartilages collapse

another view that a water-rich phase exists in the form of submicroscopic droplets interspersed in a more dense but still liquid or semiliquid colloid-rich phase; according to this view, the interstitial fluid is that portion of the intercellular fluid which retains its ability to move readily in and out of the two phases. This mobile fluid is responsible for the transport of dissolved substances between blood and cells, in both directions; it no longer seems necessary to postulate interstitial fluid as a separate phase, distinct from the solids of the ground substance.

Extension of these concepts to bone offers additional difficulties. Beginning with preosseous tissue, it has been proposed that the collagen fibrils are formed from tropocollagen in the dense phase of the ground substance, and that these are accompanied by the first and smallest crystals of the bone mineral. As both fiber formation and calcification reach their maximum, the possibilities of movement of fluid, and especially of the denser phase, become sharply limited; the ground substance of bone is in a more nearly static condition, with the movement of fluid limited to that containing small ions or molecules, capable of penetrating between the crystals of mineral.

CHEMISTRY OF GROUND SUBSTANCE

The ground substance of connective tissue is characterized by its content of polysaccharides containing hexosamines, or amino sugars, and by its staining reactions. The polysaccharides incorporating hexosamines are known as mucopolysaccharides. Of these the best studied are *hyaluronic acid* and the *chondroitin sulfates*, of which three are known—A, B, and C, respectively. The mucopolysaccharides found in bone include chondroitin sulfate A, keratosulfate, and hyaluronate, together with additional unidentified sulfated fractions. On hydrolysis, or on digestion by testicular hyaluronidase, chondroitin sulfate A yields glucuronic acid, with a repeating unit of chondrosin. Hyaluronic acid yields glucuronic acid with a repeating unit of hyalobiuronic acid. Keratosulfate is a polymer of galactose, N-acetyl glucosamine, and sulfate. The physiologic significance of the mucopolysaccharides, isolated from a wide variety of sources, is but little understood.

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The plant proteases—papain, bromelin, and ficin—have been useful for the study of ground substance. When crude or inactivated papain is injected into a young rabbit the ear cartilages collapse

and within a few days resynthesis and redeposition occur, and the rabbit's ears again become erect. During the phenomenon of collapse there occurs an uncoupling of chondroitin sulfate from the protein to which it is bound in cartilage and connective tissue. This results in complete loss of metachromasia from the cartilage; the metachromatic material accumulates in the adjacent connective tissue, is picked up by the lymphatics, and then appears in the blood and urine. Cortisone prevents the reconstitution of chondroitin sulfate-protein complex. Similar depletion of basophilia, owing to removal of chondroitin sulfate from growing cartilage or new bone, occurs *in vitro* when vitamin A is added to culture media containing chick embryo limb buds.

Lathyrism, a syndrome induced in man by diets containing large amounts of peas of the genus *Lathyrus*, and characterized by spastic paraplegia, pain, hypesthesia, and paresthesias, has been recognized in man for centuries. It occurs sporadically, especially in times of famine, in India, North Africa, and Southern Europe. No skeletal lesions have been observed. A skeletal form of lathyrism produced in rats by diets containing sweet peas (*Lathyrus odoratus*) has been studied intensively by Ponseti; the condition as it occurs in man and that produced in animals appear to be two completely different diseases, caused by different species of Lathyrus peas; that produced in rats has been designated as *odoratism*, to differentiate it from human lathyrism.

Odoratism, as observed in rats, is caused by *aminonitriles;* a number of such compounds have been found in several varieties of sweet peas; these compounds are absent from the legumes of common consumption as well as from the peas that cause human lathryism. Odoratism in rats is characterized by kyphoscoliosis, exostoses, hernias, and lameness of the hind legs, with other skeletal deformities. The aminonitriles are tissue specific, affecting mesodermal tissues, and appear to have a special affinity for the ground substance. Actively growing epiphyseal plates and certain tendon and muscle insertions are markedly affected by these compounds.

The ground substance of bone, as well as of other connective tissues, is responsible for certain staining reactions: *metachromasia* and a positive *periodic acid–Schiff reaction* (PAS). Metachromasia

is the phenomenon, described by Ehrlich, of the staining of certain tissue components in a color different from that of the dye solution itself. Thus, when cartilage is stained with toluidine blue, part of the tissue displays a reddish color. As chondrocytes mature and hypertrophy the large β-metachromatic (purple-staining) granules differentiate into small γ-metachromatic (pink-staining) granules. Metachromasia is believed to be due to the presence of free electronegative surface charges. The change in type of metachromasia of the granules reflects an increase in the number of free sulfate or carboxyl groups in the mucopolysaccharides, with possibly also a rearrangement of these groups. The ground substance of bone stains metachromatically, but only after decalcification; this reaction is associated with the mucopolysaccharide components, and more particularly with the state in which they are present. When depolymerization of chondroitin sulfate is brought about by the action of hyaluronidase, metachromasia is lost.

The *Hotchkiss procedure*, resulting in the PAS reaction, consists in treatment by periodic acid, followed by staining with fuchsin sulfite (leucofuchsin). The periodic acid oxidizes to aldehyde groups, with cleavage, adjacent hydroxyl or hydroxyl-amino groups in carbohydrate molecules. The colorless leucofuchsin then forms a polysubstituted dye compound with the aldeyhde group, pale pink to purple-red in color. The intensity of the color depends on the number of reacting aldehyde groups formed by periodic acid oxidation. Free aldehydes do not contribute to the staining reaction; simple sugars, being water-soluble, also do not affect the color of the sections. The staining reactions of the ground substance are referable (1) to the content of insoluble polysaccharides in the tissue and (2) to a change in their state. Ground substance containing highly polymerized mucopolysaccharides stains a pale pink color; this is interpreted to mean that it has relatively few reactive groups available for visualization. Depolymerization leads to an increase in color, owing to the release of a greater number of reactive groups.

When these criteria, in part based on assumptions, are applied to the study of bone, it is concluded that the ground substance of fully-formed bone, being barely stainable by the PAS reaction, con-

tains only highly polymerized mucopolysaccharides. When bone is being deposited or resorbed rapidly, the stain becomes more intense; this indicates a lesser degree of polymerization. In the epiphyseal cartilage disk of growing bones of rats, the matrix of hyaline cartilage stains a pale pink color. As the zone of provisional calcification is approached, the matrix takes on more color; and where the cartilage cells are markedly hypertrophied, the matrix is stained a brilliant purple-red, persisting when the cartilage is incorporated in the spicules of spongy bone. It has been suggested by Gersh that the property of calcifiability is dependent upon the state of polymerization or depolymerization of the mucopolysaccharides of the ground substance; this is an attractive hypothesis, but final decision must await further evidence. It is certain that calcifiability does not depend solely upon the content of mucopolysaccharides; otherwise hyaline cartilage, being the richest source of chondroitin sulfate, should be subject to calcification.

RETICULIN

The composition of reticular fibers, present in small quantities in the organic matrix of bone, occupies a position intermediate between collagen and the mucopolysaccharides of the ground substance. Quantitative data are lacking, but reticulin is believed to be a glycoprotein, i.e., a polysaccharide in combination with protein containing fucose, mannose, and galactose, and possibly hexosamine. The relationship between reticular and collagenous fibers is imperfectly understood. The fibers in the skin of newborn rats have an affinity for silver and are, by definition, reticular fibers. Electron microscopy of these fibers reveals the periodic cross-banding characteristic of collagen. With increase in age there is a decrease in the proportion of argyrophilic fibers, without any demonstrable difference in structure. Similar observations on reticulin from the organic matrix of bone have not been reported.

WATER OF BONE

The water content of bone varies with the species of the animal, with age, with the nutritional state of the individual, and with the nature of the bone tissue under examination. As a representative

standard for the compact bone of an adult dog, Robinson has selected a figure of 3.68 per cent of water *by weight*, with 72.17 per cent of mineral and 24.15 per cent of organic matter. Owing to the differences in density, the corresponding figures, in terms of the volume of the sample of bone, are 8.2 per cent for water, 53.62 per cent for mineral, and 38.18 per cent for organic matter. Of the water present, some 10–15 per cent of the total volume is in the spaces in the bone occupied by canalicules, haversian canals, and osteocytes; 85–90 per cent is in the organic matrix, including the collagen fibers and ground substance, and in the hydration shells of the crystals of bone mineral. Drying at 100° C. removes the water of constitution of the collagen and of other organic matter, but not the water of the bone crystals. The water of compact bone is so firmly bound that little or none can be removed from finely powdered cortical bone by enormous centrifugal forces—forces sufficient to strip all mechanically held water from the crystals.

Perhaps the most important concept concerning the water of bone is that while the organic matter of normal compact bone remains relatively constant in relation to volume, calcification of new haversian systems occurs by replacement of water by crystals of bone mineral. The space available for mineral is thus the greater part of that occupied by water; crystallization proceeds until there is no space left for further expansion. As crystals form and grow in a fixed volume, by displacement of water, the spaces between the crystals become smaller and smaller; eventually a state of maximal, diffusion-locked mineralization is achieved, owing to inadequate space for the diffusion of new ions.

In spite of the reduction in the ability of ions to diffuse in bone, incident to maximal mineralization, it has been found that deuterium, when administered in the form of heavy water, D_2O, is able to permeate all areas of bone, and to exchange freely, but slowly, with the H^+ of water. There is thus restricted diffusion of even the D^+ ion. The slow movement of Ca^{45} into the stable portion of bone, which characterizes long-term exchange, must take place in part by diffusion, where there is sufficient space for the ions to move between crystals, and in part by intracrystalline exchange. The

packing of the water spaces with crystals differentiates calcified from soft tissues and is doubtless responsible for the relative inaccessibility of the bone mineral to ion exchange.

FLUORESCENCE IN BONE

Bone salt produces a light-blue fluorescence in ultraviolet light. This physical property can be used to detect calcium salt in new bone in microscopic quantities. Owing to a complex interaction in newly formed bone it is possible to change the fluorescence from blue to red, yellow, and other colors. In this way, new bone can be differentiated from older deposits of bone. John Belchier, in 1736, reported that madder stained the new bone in the skeletons of birds and pigs fed bran soaked in madder; the color depends on ruberythric acid which yields alizarin on hydrolysis. Synthetic alizarin is 1,2-dihydroxyanthraquinone; fluorescent solutions indicate the presence of unchanged 2-anthraquinone sodium sulfonate. Alizarin as well as tetracycline, chlortetracycline, and oxytetracycline have been found to fluoresce brightly in the new bone of animals and man examined under ultraviolet light. Alizarin Red S fluoresces deep red; chlortetracycline, orange; and tetracycline, light yellow. Quercetin, a flavine derivative, fluoresces an intense golden yellow. Other substances, e.g., porphyrins, carotinoids, lipofuchsins, and oxidized cytochrome, also produce a red or orange fluorescence, but appear in soft tissue as well and are not useful for experimental work on bone (see Frontispiece).

CHAPTER V

Crystal Structure and Chemical Composition of Bone Mineral

The hardness of bone, its outstanding characteristic, results from its content of a mineral, deposited in the organic matrix. The mineral, commonly called the bone salt, and the organic matrix, together with their associated water, make up the interstitial substance of bone. The mineral fraction consists mainly of Ca^{++}, $PO_4^{\equiv}$, OH^-, $CO_3^=$, and citrate, with an inclusion of small amounts of other ions, especially Na^+, Mg^{++}, and F^-. The crystal structure and chemical composition of the bone mineral have been under investigation for more than one hundred years, and they remain a subject of intensive study.

Certain statements may be made about the bone mineral, to serve as a guide to its composition, structure, and behavior: (1) the crystals are ultramicroscopic in size, extremely minute, and hardly more than of colloidal dimensions; (2) the bone salt is not a single, homogeneous chemical individual; (3) the basic atomic structure is well-known, chiefly from X-ray diffraction patterns, and is that of an apatite mineral, protoypes of which are common in nature; (4) the chemical composition is not as accurately known, partly because of its variability and partly because of the difficulty of differentiating between (*a*) the constituents of the basic structure, (*b*) the ions having only a surface relationship to the crystals, and (*c*) those possibly admixed with the apatite crystals, in a separate phase; (5) the structure and composition of the mineral correspond most closely to that of hydroxyapatite in all vertebrates studied; the mineral component, however, includes carbonate and citrate in substantial amounts; (6) various substitutions and exchanges of

ions occur in the crystal structure; (7) dissolution and recrystallization may occur; (8) the minute crystals and the voids and discontinuites in the mineral expose large surfaces to the fluids of the body; (9) the ions held by adsorption or by substitution at these surfaces, as well as those incorporated within surface unit cells, with unshared sides, have a considerable influence upon the total composition of the mineral; (10) the surface ions are subject to rapid exchange with the fluids bathing the crystals; and (11) the reactivity of the mineral varies with its age and with its immediate *environment.*

CRYSTALS OF BONE MINERAL

The crystals of the mineral of bone have long been known to be extremely minute—too small to be seen with the light microscope. While it was first shown by X-ray scatter patterns that the crystals are of the order of a few hundred A in size, current knowledge of the size and shape of the individual crystals is dependent mainly on electron microscopy. This method has its limitations, but the resolving power of the electron microscope has been increased, and the crystal of bone mineral is within the limits of its resolution.

Early reports of direct observation of crystal size and shape, by means of the electron microscope, described the crystals as minute tablets of a few hundred A in length and breadth, and a thickness of only 20–50 A; later reports were of rodlets or hexagonal prisms. These descriptions were supported by low-angle X-ray scatter patterns, from which measurements of approximately 200 A in length and 75 A in diameter were calculated. Current estimates are in good agreement, to the effect that the diameters of rod-like crystals range from 25 to 75 A, with an average of 50 A. There have been, however, differences in estimates of the dimensions in length. In a study of the crystals in the parietal bone of one- to two-week-old mice, Molnar postulates that the crystals are composed of chains of microcrystals in an end-to-end relationship from an original length of 50 A up to an unlimited length. The rod-like crystals so formed frequently extend over several major periods of the collagen fibrils; some were traced up to a length of 3,000–4,000 A; all exhibited sub-units of about 50 A.

On the basis of evidence now available, the crystals may be visualized as rod-like, with diameters of about 30 A, and of indeterminate length, oriented in the long axis of the collagen fibrils, and with a special relationship to the cross-banding of the fibrils. The possibility that many of the crystals are prism-like, with the smallest dimension of the order of 20–30 A, is not excluded; the

FIG. 11.—Electron micrograph of a section of undecalcified bone, in the long axis of the fibers. Needle-shaped crystals in the same axis appear in relation to the cross bands. In some instances they extend over the intervals between the cross bands. ×200,000. (*Reproduced by courtesy of M. J. Glimcher, A. Hodge, and F. O. Schmitt.*)

size and shape of the crystal have a marked influence upon its surface chemistry, upon the exchangeability of its surface ions, and possibly upon the ease with which it goes into solution (Fig. 11).

INTERNAL STRUCTURE OF CRYSTALS

Much of the present knowledge of the internal structure of the microcrystals of the bone mineral stems from interpretation of the X-ray diffraction pattern of the crystallites of bone, as compared with patterns of relatively pure samples of synthetic or naturally occurring crystals of minerals of known composition. When a monochromatic X-ray beam is passed through a regular array of atoms,

e.g., a crystal, a diffraction pattern is produced, related to the atoms in the material, and more especially to their spatial arrangement. With a film near the irradiated sample it is possible to measure the angles between the incoming X-ray beam and the outgoing diffracted beams. Data may thus be obtained concerning the arrangement of atoms within the crystal, including the distances between the atoms, and also concerning the orientation of the crystals.

It is firmly established that the mineral constitution of bone consists chiefly of crystals of a compound of calcium and phosphate with the structure of an apatite. The apatite series contains a number of compounds which have their constituent ions arranged similarly in a three-dimensional symmetry pattern. The apatites usually have: (*a*) divalent cations (Ca^{++}, Pb^{++}, Sr^{++}), (*b*) tetrahedral anionic radicals ($PO_4^{\equiv}$, $SiO_4^{\equiv}$), and (*c*) electronegative anions (OH^-, F^-), all in a hexagonal symmetry array characteristic of this isomorphous series. Certain ions can be substituted one for the other, e.g., Pb^{++} for Ca^{++}, F^- for OH^-, without disturbing the symmetry of the structure, although the distance between ions may change, owing to the differences in ionic size. X-ray diffraction techniques can easily detect changes of the order of 0.5 per cent in interatomic differences resulting from isomorphous substitution.

X-ray diffraction, together with chemical analysis, has brought about general agreement that the basic structure of bone mineral is some form of hydroxyapatite, of which the prototype is:

$$Ca_{10}(PO_4)_6(OH)_2 \text{ (hydroxyapatite).}$$

It is therefore possible to treat the internal structural characteristics of the bone mineral with some degree of certainty; the uncertainties in the exact chemical composition will be treated in a later section.

UNIT CELL

The internal structure of the hydroxyapatite crystal is best understood in terms of the *unit cell*. This is a conceptual configuration, having no independent existence. It is the smallest expression of the ions found in the same ratios and in the same spatial re-

lationships in which they are present throughout the entire crystal. Imaginary lines, connecting the ions, drawn in an arbitrary but regular way, outline the unit cell; when extended through the crystal structure they result in a three-dimensional lattice—the *crystal lattice.*

The crystal structure of hydroxyapatite may be represented in two different ways. In a cross-section of the crystal, perpendicular to the long axis, it may be represented as a series of contiguous hexagons (Fig. 12). At each intersection there is a Ca^{++} ion, shared between three hexagons, and surrounded by $PO_4^{\equiv}$ ions, arranged in

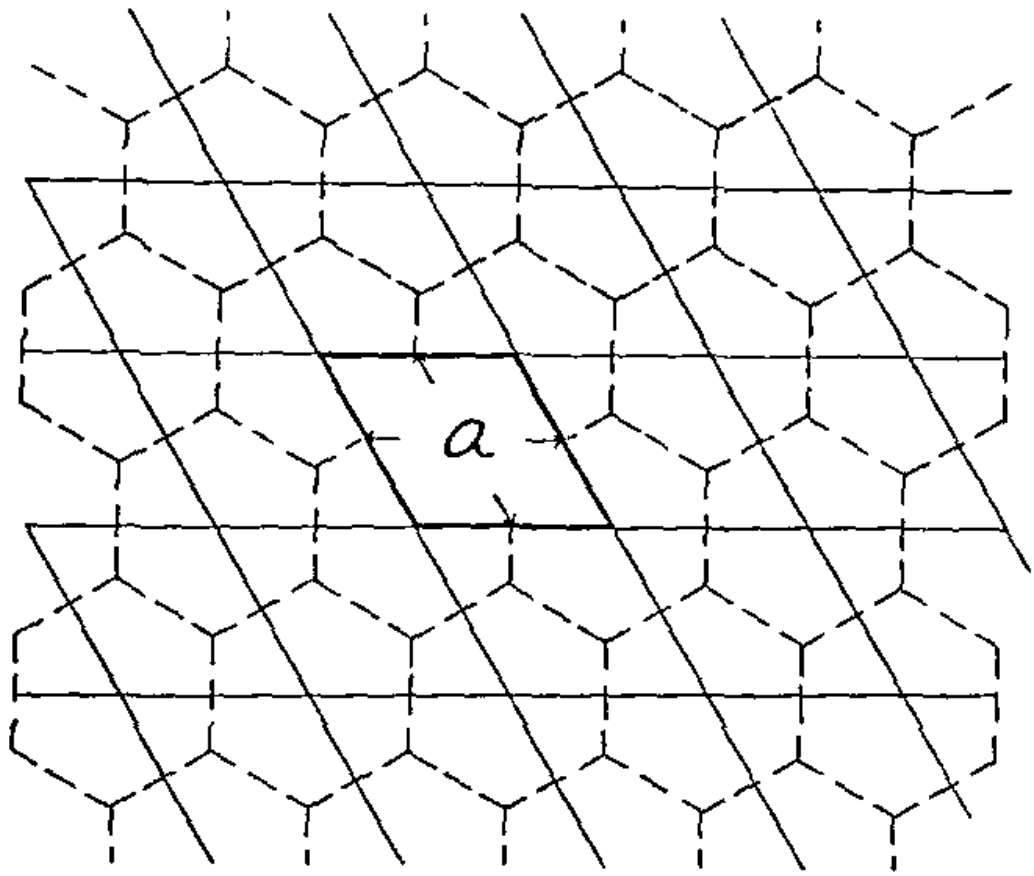

FIG. 12.—*Diagram of a cross-section of a crystal of bone salt, to illustrate internal structure.* The parallelograms represent the unit cells of the crystal, these are conceptual, rather than structural, units, since they illustrate only the arrangement of constituent ions in space. The hexagons represent a *honeycomb-like arrangement of* ions, oriented with the long axis of the crystal. At each intersection of the hexagons is a Ca^{++} ion. The tunnels are lined with Ca^{++} ions and packed with $PO_4^{\equiv}$ ions, with a column of OH^- ions in the center. Scale ×20,000,000 (2 mm. = 1 A). *The parallel sides of the unit cells (a-axes) are approximately 0.43 A in length.*

a complex pattern. At the center of the hexagon there is an OH^- ion, shared by three Ca^{++} ions. The two OH^- ions of hydroxyapatite are superimposed on each other; three of the six Ca^{++} ions forming the hexagon share one OH^- ion; the other three share the next.

The second method represents the unit cells. The points corresponding to the OH^- ions in a cross-section of the crystal may be connected in a two-dimensional diagram as a series of diamond-shaped parallelograms, with angles of 120° and 60° at the intersections. When extended in a third dimension, this forms a parallelepiped, or a six-sided right prism, four of whose faces are rectangles, with two faces as parallelograms. Each of these geometric figures is, by convention, a unit cell of the crystal lattice. The parallelograms of a unit cell of hydroxyapatite have four equal sides, measuring approximately 9.43 A in length, representing the *a*-axes of the cells (Fig. 12). The third dimension, or *c*-axis, measures approximately 6.88 A.

The *c*-axes of the unit cells are oriented in the long dimension of the crystal of hydroxyapatite. When a diagram of the lattice of an ideal crystal is viewed parallel to the *c*-axis, there are continuous columns of calcium and oxygen, arranged at the intersections of the hexagons, and columns of OH^- ions at the intersections of the parallelograms representing the unit cells. From this point of view there is a sort of honeycomb arrangement, oriented in the long axes of the crystals. The columns of calcium and oxygen, at the intersections of the hexagons, give the form to the honeycomb; these columns are surrounded by phosphate, with a column of OH^- ions in the center of each hexagon. In addition, there is a screw axis, running spirally around the columns of OH^- ions; Ca^{++} ions, in addition to those at the intersections of the hexagons, are symmetrically arranged with respect to this axis, and are also accompanied by phosphate ions.

VARIABILITY IN STRUCTURE

The descriptions just given refer to perfect or ideal crystals of hydroxyapatite. The real crystals, as they occur in bone, are neither perfect nor ideal. Many discontinuities are formed within the struc-

ture of a single crystal as it grows, and others form with time. At the surfaces of the discontinuities, which are irregular and occur at random, impurities may exist; in fact, if such impurities are of sufficient magnitude, they may be the cause of the discontinuity. Moreover, substitutions occur in the unit cells; not all unit cells are identical in composition or even in structure.

The crystals of the bone salt are only a few unit cells thick. The surface is therefore large in proportion to the mass, and 1 gm. of the mineral is reported to have a surface area in excess of 100 square meters. Calculations from these figures lead to the rather startling conclusion that the total surface area of the bone crystals in the skeleton of a man weighing 70 kg. exceeds 100 acres! The implications of the fact that such a large area is bathed by a few liters of body fluid are many.

Because of the small size and the thinness of the crystals, one-half to two-thirds of the unit cells are located in the surface, possess one or more unshared sides, and are subject to special surface conditions. These conditions have created difficulties in characterizing the bone mineral and are as yet not fully understood. When taken into account, it becomes clear that the nature and structure of the bone mineral and the dynamics of its behavior are largely problems in surface chemistry.

Discontinuities in the bone mineral are not limited to the interior of the crystals; the crystals themselves are discrete; the spaces between them are filled with the organic matrix, water, and the solid constituents not included in the crystal structure. If one considers bone from which the organic matter and water have been removed, it is as though the bone mineral itself is built up of bricks and mortar rather than as a continuous mass of a homogeneous substance. It thus resembles a brick wall, rather than a monolith, but with a pattern peculiar to itself (*Fig. 13*). The bricks are the crystals of apatite; the mortar consists chiefly of citrate and carbonate, with an admixture of other ions. In the living animal the structure is plastic rather than rigid; the organic matrix and the intercellular fluid are also in the spaces between the crystals; the collagen fibers act as reinforcing strands in the loosely assembled intercrystalline

portion of the structure. From studies made with the electron microscope it is reported that the crystals lie in the ground substance, forming a ring-like periodic pattern around the collagen fibers. The intervals between the rings are about 640 A, corresponding to the intervals between the cross-banding of the fibers; the

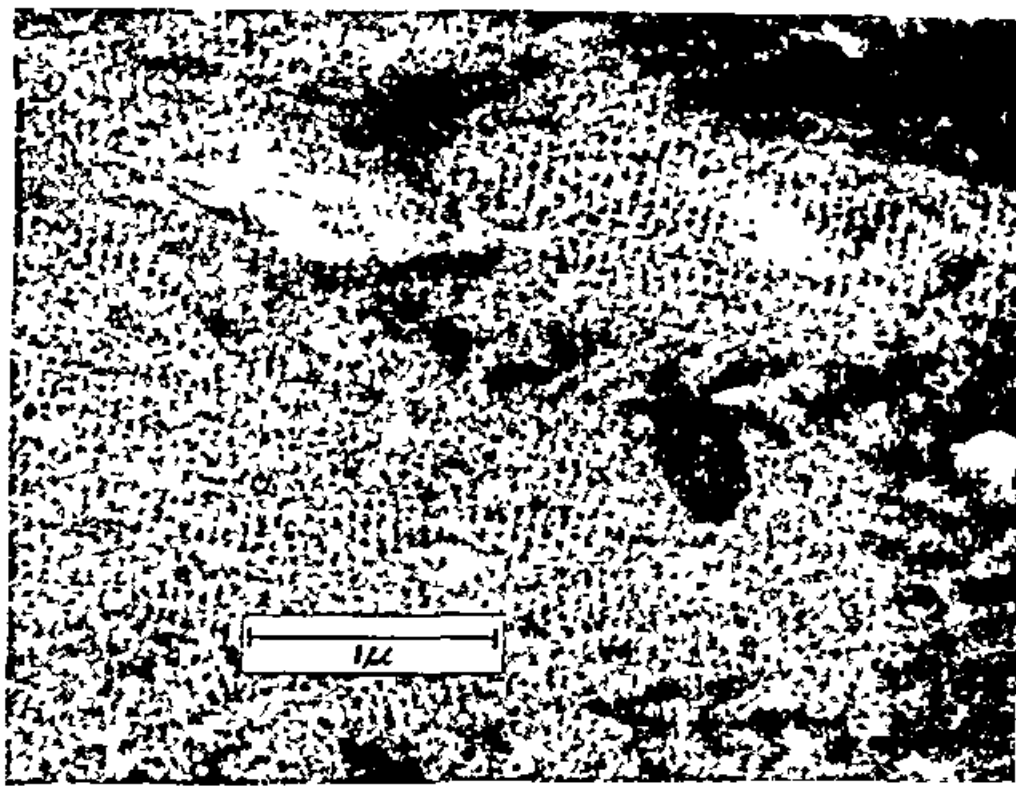

FIG 13.—Electron micrograph of a section of an undecalcified human rib, to illustrate pattern of arrangement of crystals of bone mineral in relation to collagen fibers The underlying collagen is barely visible, but the crystals are easily seen in the regularly arranged bands corresponding to the main collagen striation. The longitudinal direction of the fibers is horizontal. (From Fig. 1, Neuman and Neuman, *Chem. Rev*, 53:4 *[courtesy of R. A. Robinson and M. L. Watson]*. Reproduced by courtesy of the publishers)

crystals have a definite relationship to the cross-banding (Fig. 13). Some of the mineral is deposited *within* the collagen fibers; in this location it may be in a non-crystalline state. Except for these spatial arrangements, the relationships between the bone mineral and the organic matrix are poorly understood.

It is convenient to consider the structure of the bone mineral as being made up of a large number of similar, but not identical, com-

plexes. These complexes are not solely the crystals of apatite, although much effort has been expended in attempting to understand the mineral in these terms. Instead, each structural complex may be regarded as including interior unit cells wholly within the crystals; surface unit cells, sharing some of their sides with interior cells; ions bound by surface forces; and a surface hydration shell, containing ions in equilibrium both with the surrounding medium and with the surfaces.

CHEMICAL NATURE OF BONE MINERAL

It has already been noted that the bone salt is not a single, homogeneous chemical individual; that the chemical composition is not accurately known; that various substitutions and exchanges of ions occur in the structure; that dissolution and recrystallization may occur; and that there are voids and discontinuities in the crystals. All of these factors make for an unsatisfactory situation with respect to the chemical characterization of the bone mineral, even though its structure, corresponding closely to that of hydroxyapatite, is well established.

ELEMENTARY ANALYSIS

Armstrong and Singer have furnished an unpublished summary of their analyses of a single sample of dry, fat-free bovine cortical bone. The standard deviations represent the variations in the analyses, and do not refer to variations of composition between various samples of bone (Table 1).

The most important single value in this tabulation, for our purposes, is the molar Ca/P ratio of 1.688; this corresponds very closely to the theoretical ratio for hydroxyapatite, of 1.667. Values for another single sample of bone as found by Bogert and Hastings differed with the previous treatment of the sample, but were in the range of 1.70 to 1.80 for untreated bone and bone extracted with water or alcohol. After more vigorous treatment to remove the organic matter, including ashing at such temperatures as to cause little or no loss of carbonate, the range was consistently between 1.57 and 1.61. If allowance is made for the Ca^{++} required to neutralize the anionic constituents of the mineral, chiefly carbonate, as

Bogert and Hastings and Neuman and Neuman have done, the residual molar Ca/P ratio is then approximately 1.5, or considerably less than the theoretical value of 1.67. The bone mineral has been compared with similar apatites, synthetically prepared, and deficient in divalent cations. The calculations of the residual Ca/P ratio in bone mineral do not take into account the cations other than Ca^{++} that are available for neutralization of the anionic con-

TABLE 1

COMPOSITION OF DRY, FAT-FREE BOVINE CORTICAL BONE

	Per Cent	mEq/gm
Cations		
Calcium	27 24 ±0 02	13 59 ±0 01
Magnesium	0.436±0 009	0 358±0 008
Sodium	0 731±0 015	0.318±0 007
Potassium	0 055±0 0009	0 014±0 0002
Total	....	14 28
Anions*		
Phosphorus	12 47 ±0 013	... .
as $PO_4^{\equiv}$	.. ...	12 08 ±0 012
Carbon dioxide	3 48 ±0 022	.....
as $CO_3^{=}$	. . ..	1 58 ±0 010
Citric acid	0 863±0 004	.....
as $Cit^{\equiv}$	. . ...	0 135±0 0006
Chloride	0 077±0 004	0.022±0 001
Fluoride	0 072±0 003	0 037±0 002
Total	..	13 85
$\frac{\text{mEq cations}}{\text{mEq anions}}$ ..	. . .	1 031
$\frac{\text{mMCa}}{\text{mMP}}$.	. .. .	1 688

stituents; their inclusion in the calculations would tend to raise the Ca/P ratio toward the theoretical value of 1.67.

There is no doubt about the ability of certain other cations to substitute for calcium in the structure of the bone mineral; this occurs by isomorphous substitution and has been demonstrated in living animals, as well as in synthetic hydroxyapatites, for strontium and for lead; radium also substitutes for calcium. Sodium is believed to substitute for calcium, as well as the hydronium ion, H_3O^+. There is no evidence that potassium substitutes for calcium in the bone mineral, and the position of the divalent magnesium ion, Mg^{++}, is doubtful; it has been suggested that the magnesium

found in analysis of the mineral constituents of bone may be in the form of the complex ion, $MgOH^+$. As to anions, the most common substitution is that of F^- for OH^-. Fluorapatites occur in nature; the occurrence of F^- in the bone mineral represents a contamination. There has been no suggestion that citrate, found in the mineral of bone in substantial amounts, can be a structural constituent of the apatite crystal; the dimensions of the citrate ion, $C_6H_5O_7^{\equiv}$, are too large for isomorphous substitution. Citrate is regarded as a separate phase, with a special relationship to the surfaces of cyrstals.

CARBONATE OF BONE

Carbonate is universally present as a constituent of the mineral of normal bones and teeth; its position in the mineral is still in debate. A carbonate-containing apatite, dahllite, exists in nature and is described as a carbonate hydroxyapatite. Similarly, francolite has been characterized as a carbonate fluorapatite. These have attracted attention because of possible analogies between their composition and crystal structure and that of bones and teeth. Prior to 1937 it was believed that $CO_3^=$ could substitute for $2\ F^-$ or $2\ OH^-$, thus forming a true carbonate apatite, but this is no longer accepted. Instead, there is now the proposal, advanced and strongly supported by McConnell, that $CO_3^=$ substitutes isomorphically for $PO_4^{\equiv}$ in the minerals, francolite and dahllite. McConnell concludes that dental enamel is composed of a single mineral, carbonate hydroxyapatite (dahllite), and states that there is no valid reason for supposing that the crystal structure or crystal chemistry of bone is significantly different. Against this is the view that $CO_3^=$ is present in the mineral of bone and teeth only in surface positions, where it substitutes for $PO_4^{\equiv}$; the evidence is summarized by Neuman and Neuman. Both views provide for substitution of $CO_3^=$ for $PO_4^{\equiv}$; agreement has not been reached as to whether this substitution occurs within the lattice structure, on the surfaces, or both. In any case, the bone mineral is to be regarded as a substituted hydroxyapatite, with the structural characteristics of the latter.

Without attempting to resolve the differences of opinion concerning the location of carbonate in relation to the structure of hydroxyapatite, it may be said that, in common with certain other

minerals, a carbonate hydroxyapatite does not lend itself to stoichiometric formulation, since CO_3^- does not substitute for $PO_4^{\equiv}$ in any fixed proportions, or in any special pattern. Instead, McConnell has supplied the following as a type of formula used to represent minerals with constituents in varying proportions:

$$Ca_{10-x}O_{2+x}H_{2+3x}(PO_4)_{6-3x}(CO_3)_{4x} \text{ (carbonate hydroxyapatite)},$$

which reduces to conventional hydroxyapatite when $x = 0$, and when $x = 1$ to the following:

$$Ca_9O_3H_5(PO_4)_3(CO_3)_4 \text{ (carbonate hydroxyapatite)}.$$

However, there is no evidence that natural substances ever have x as great as 1.

There has been little evidence to show that random substitution of $CO_3^=$ for $PO_4^{\equiv}$, either within the apatite crystal or on the surface, is of physiologic importance; this question would assume much significance if it could be shown that the presence of $CO_3^=$ is essential for the mineralization of bone. McConnell and his coworkers have shown that a mineral substance similar to that of bone, and with a diffraction pattern virtually identical with that of the mineral of calcified shark's cartilage, could be produced *in vitro*, under the conditions of their experiments, only in the presence of available carbon dioxide. Carbonic anhydrase catalyzes the formation of this mineral and is essential to its deposition when the carbon dioxide content of the medium is low; inactivation of the enzyme by sulfanilamide prevents mineralization under these conditions. Hydroxyapatite, on the other hand, has been produced in essentially pure form by others, without either carbon dioxide or carbonic anhydrase. Sobel has shown that the carbonate content of the bone mineral depends upon the Ca/P ratio of the serum, as influenced by dietary Ca/P ratios and levels. The skeleton participates in buffering the carbonate (bicarbonate) of the blood and tissue fluids; whether this depends upon the position of the carbonate ion in the bone mineral has not been shown.

HYDRATED TRICALCIUM PHOSPHATE

The formulation of hydroxyapatite calls for a molar Ca/P ratio of 1.667, as already noted; actual ratios in the bone mineral have

been reported as lower, or approximately 1.5. This deviation from the theoretical ratio is in part responsible for Dallemagne's postulation of a hydrated tricalcium phosphate (alpha tricalcium phosphate), instead of hydroxyapatite. Dallemagne proposed the formulation:

$$3Ca_3(PO_4)_2 \cdot H_2(OH)_2 \text{ (alpha tricalcium phosphate)}.$$

In support of this proposal, data from ion exchange reactions are reported and interpreted to indicate that the compound is unstable; that its instability is responsible for its reactivity in the living organism; and that on isolation from its association with the organic matrix, it is transformed into the stable hydroxyapatite, identical with the substance produced by synthesis *in vitro*.

Posner has modified this view by preparing a series of calcium-deficient hydroxyapatites, the formulas of which can be written as

$$Ca_{10-x}H_{2x}(PO_4)_6(OH)_2 \text{ (calcium-deficient hydroxyapatite)},$$

and has indicated that Dallemagne's concept of alpha tricalcium phosphate is a special case of the calcium-deficient hydroxyapatites. Posner also does not accept an alternative proposal for calcium-deficient hydroxyapatites, i.e., that the hydronium ion, H_3O^+, substitutes for Ca^{++}; he proposes instead that hydrogen bonding, and not hydronium ions, is responsible for maintaining neutrality in low calcium hydroxyapatites. The substitution of other cations for Ca^{++}, e.g., Na^+, also contributes to a low Ca/P ratio; adsorption of HPO_4^- on the crystal surfaces, another possibility, is not now often referred to.

SUMMARY

The most widely-held view of the crystal structure and chemical composition of the bone mineral is that it is basically hydroxyapatite, i.e., $Ca_{10}(PO_4)_6(OH)_2$, although certain deviations from this view are also currently accepted, as noted in the introduction to this chapter. As attested by the dissenting views here recorded, it is clear that its subject matter is still in a fluid state and that no final statement can be made on the topics treated. This is a field which is still being intensively cultivated, and it may be hoped that the next few years will bring additional clarification.

CHAPTER VI

Dynamics of Bone Mineral

The preceding chapter has dealt with the crystal structure and chemical composition of the bone mineral, largely in static terms. This chapter will be concerned with its dynamics, including such topics as nucleation, crystal formation, crystal growth, solubility, and reactivity. The subject matter of this chapter, as well as that of the former chapter, is treated at length in a monograph, *The Chemical Dynamics of Bone Mineral*, by Neuman and Neuman, published in 1958 (The University of Chicago Press), which includes analysis of the evidence for many of the views here presented in a more condensed form. Study of the dynamics of the bone mineral has been mainly concerned with calcification, including nucleation and crystal growth. In recent years, much attention has also been given to the reactivity of the mineral, and more especially to the transfer of ions between bone and blood.

SOLUBILITY RELATIONSHIPS

That the bone salt is difficultly soluble is of far-reaching physiologic significance. This property not only determines the deposition of the bone mineral; it preserves the structure and the rigidity of the bones. Moreover, it is of first importance in maintaining the balance of minerals between the skeleton and the fluids of the body.

The adult organism as a whole is normally in approximate calcium balance, i.e., the output of calcium is equal to the intake. There is a constant turnover of the bone mineral; and since the bones account for by far the greater part of the body's store of calcium, an over-all calcium balance means that the rates of deposition and of solution of the bone mineral are also in balance.

This is true even though deposition and solution of the mineral may occur at different rates in different parts of the skeleton at the same time.

It is desirable to review here the concept of the solubility product constant. An *ion product* is the product of the molar concentrations of ions in solution, relevant to the salt under consideration. The *law of mass action* requires that when an ion is present in the formula of a salt in numbers greater than one, its molar concentration in the ion product must be raised to the power corresponding to the number of ions in the formula. The ion product of $CaHPO_4$ is

$$[Ca^{++}] \times [HPO_4^{=}],$$

while the ion product of $Ca_3(PO_4)_2$ is

$$[Ca^{++}]^3 \times [PO_4^{\equiv}]^2.$$

In these expressions the brackets, [ ], are used to indicate *concentrations* in mols per liter. Application of the Debye-Hückel theory of interionic attraction would require that these concentrations be corrected by *activity coefficients;* the physicochemical activity of ions is decreased as the *ionic strength* of the solution is increased. The development of the subject of ion products without these corrections is admittedly an oversimplification; since activity coefficients remain constant at constant ionic strength, it is justifiable to omit them for purposes of comparison, when body fluids of approximately constant ionic strength are under consideration.

A *solubility product constant* is the ion product at concentrations of the ions at which the rates of solution and of precipitation of the salt are equal. The solubility product constant thus defines the conditions present at equilibrium; these are identical with those in a saturated solution containing the ions of a difficultly soluble salt.

Numerous attempts have been made to define the solubility of the bone salt in simple physicochemical terms, i.e., by the determination of a solubility product constant. These have not been successful, for several reasons. First, the concept of solubility product is meaningless without reference to a known and homogeneous solid phase. The bone mineral does not meet these requirements.

Second, equilibrium between the solid and liquid phases of hydroxyapatite is attained *in vitro* only very slowly and with great difficulty. Moreover, the results of equilibration may differ, according to whether the state of saturation is approached from supersaturation or from undersaturation. A part of this difficulty stems from the fact that the solid phase of the mineral may adsorb ions from solution. Third, the solid phase may undergo changes in composition during equilibration. Fourth, the relations between the bone mineral and the fluids bathing it are influenced by certain biologic factors, such as the state of activity of the parathyroid glands.

Levinskas has made a careful and detailed study of the solubility of a synthetic hydroxyapatite, under widely varying conditions. The reproducibility of the results and the agreement of findings, when undersaturated and supersaturated solutions were employed initially, support the belief that equilibrium was attained. Even under the rigorously controlled conditions of the experiments, however, the constant, well-characterized solid phase employed did not permit determination of a solubility product constant. It was concluded that it is impossible to determine a $K_{s\,p}$ for such examples of the apatite lattice in aqueous systems. The factors entering into this situation are reviewed in detail by Neuman and Neuman, who conclude that the blood serum is supersaturated with respect to bone mineral, but below the point of spontaneous precipitation.

Earlier, Logan and Taylor brought bone powder to equilibrium with solutions containing calcium and phosphate, and they expressed the conditions at equilibrium as an ion product, which they regarded as a solubility product constant, as follows:

$$[Ca^{++}]^3 \times [PO_4^{\equiv}]^2 = K_{s\,p\,Ca_3(PO_4)_2}$$

$$-\log K = pK_{s\,p\,Ca_3(PO_4)_2} = 23.1 \pm 0.4\,.$$

Although, for reasons as stated above, their formulation cannot be accepted as defining the solubility of the bone salt, it is still of interest and empirical value. They approached equilibrium from both supersaturation and undersaturation; moreover, they reduced the quantity of the solid phase to the vanishing point, thus minimizing

adsorption. We are retaining their values for comparison with results obtained with a biologic end-point.

More recently, MacGregor and Nordin have carried out equilibration studies with human bone powder, comparable to those of Logan and Taylor, but using different methods. In the pH range of 6.58–7.8 they obtained a relatively constant ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ whether equilibrium was approached from supersaturation or undersaturation. The arithmetic mean product observed was 4.1×10^{-27}, corresponding, in the terms of Logan and Taylor, to $pK = 26.39$. This figure approximates that obtained by Logan and Taylor when they used a high solid/solution ratio, and was reduced by them to 23.1 when they eliminated the influence of adsorption by using very small quantities of the solid phase. Since the figure obtained by MacGregor and Nordin corresponds to the ion product in normal tissue fluids at pH 6.8, they suggest that this may be the pH at the surface of the bone mineral. This subject is given further consideration in connection with the surface chemistry of the mineral.

BIOLOGIC SOLUBILITY

Much of the difficulty in expressing the conditions for crystallization of the bone salt in physicochemical terms has been due to the requirement of attaining equilibrium between the liquid and the solid phases and of determining when this end-point has been reached. To avoid the necessity of equilibrium, the concept of *biologic solubility*, with the use of a biologic end-point, was introduced some years ago. Such an end-point, in order to be meaningful, may be described in terms of an ion product. It therefore has the characteristics of a solubility product constant, with the exception that the criterion for arriving at the constant is the phenomenon of calcification itself. The limit of biologic solubility is the minimum ion product at which calcification will occur.

If young growing rats are fed diets deficient in phosphate and in vitamin D, the bones continue to grow, but calcification ceases. There follows an overgrowth of cartilage, accompanied by the appearance of uncalcified bone matrix—the osteoid tissue. This condition, characterized by a failure of calcification to keep pace with

growth, is *rickets*. If the deficiencies in the diet are corrected, calcification begins again, both in the cartilage matrix and in the osteoid tissue. This occurs also in cartilage matrix *in vitro* when slices of rachitic cartilage are incubated under favorable conditions, and both the locus of calcification and the product obtained are comparable to those observed in the living animal. Most of our information concerning the dynamics of calcification is based on *in vitro* studies, but enough observations have been made *in vivo*, both in rachitic children and in experimental animals, to make it appear that the results obtained *in vitro* are applicable to the living organism. Calcification of the cartilage matrix *in vitro* has been used as the end-point for biologic solubility.

CALCIFICATION: HUMORAL CONDITIONS

Calcification, in the systems with which this book is concerned, is essentially the deposition, within a soft organic matrix, of a difficultly soluble compound of calcium, resembling the hydroxyapatite described in the foregoing chapter. For calcification to occur, certain fundamental conditions must be present. These divide themselves naturally into (1) humoral and (2) local conditions. The *humoral conditions* embrace the supply and transport of the minerals necessary to calcification and their delivery to the locus of calcification in the concentrations required. The local conditions include whatever it is that differentiates a *calcifiable* from a noncalcifiable tissue. This is at times referred to as the *local factor*. Since, however, several interrelated factors, enzymatic and nonenzymatic, appear to be involved, it will be convenient to refer to these collectively as the *local mechanism*. In considering the humoral conditions for calcification, it is apparent that in spite of the difficulties in defining the solubility relationships of the bone salt, its deposition and its maintenance in the solid state depend primarily upon its solubility.

Howland and Kramer, in 1923, in the absence of any method for determining either calcium or phosphate ion concentrations in the plasma, introduced a simple empirical product, Total Calcium *times* Total Phosphate, and found that the presence or absence of

rickets in infants could be correlated with this product. Further refinement of this formulation has since been possible, and it has now a sound, although incomplete, basis.

Of the 1–2 mM per liter of phosphorus in the plasma, as inorganic phosphate, approximately 85 per cent is in the form of the divalent ion $HPO_4^{=}$, while 15 per cent is $H_2PO_4^{-}$ and only 0.0035 per cent is present as $PO_4^{\equiv}$. Ca^{++} and $HPO_4^{=}$ may combine in a one-to-one ratio, and both are present in the fluids of the body in appreciable concentrations. For these reasons it should not occasion surprise that since the publications of Howland and his associates the findings have been more consistently related to the ion product $[Ca^{++}] \times [HPO_4^{=}]$ than to any other combination.

In view of these considerations, the concept of biologic solubility has been made use of in an attempt to determine whether the ion product of $CaHPO_4$ or that of $Ca_3(PO_4)_2$ is critical for calcification. The results are presented, with the reservation that the theoretical significance of the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ is not established.

Over a wide range of H^+ concentration, pH 6.0–8.5, and of total phosphate concentration, 3–80 mM per liter, and with the Ca^{++} concentration kept constant, the minimum ion products at which calcification occurred in rachitic bone slices were determined. These points were plotted against total phosphate concentrations and pH (Fig. 14). On the same plot were drawn curves representing $pK_{sp\,CaHPO_4} = 5.47$, and $pK_{sp\,Ca_3(PO_4)_2} = 23.1$, at varying concentrations of phosphate and varying pH. On the acid side of pH 7.3, the points at which calcification just occurred followed the curve for the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, while on the alkaline side, the points coincided closely with the curve for the ion product $[Ca^{++}] \times [HPO_4^{=}]$.

The significance of the results on the acid side of pH 7.3 is not clear, partly because of the uncertainties concerning the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$. In addition, the ionic strength of the solutions increases rapidly with increases in concentration of total phosphate. This has the effect of decreasing the activity coefficients of both Ca^{++} and $PO_4^{\equiv}$; and if the proper corrections could be made, this might have the effect of bringing the experimentally determined

points close to the curve representing $pK_{s\,p\,CaHPO_4}$. In spite of this uncertainty, the points are of interest because of their internal consistency.

On the alkaline side of pH 7.3 it appears that the ion product for $CaHPO_4$ is critical for calcification and that the end-result is but little affected by an increase in alkalinity from pH 7.3 to pH 8.5. The fact that increasing the alkalinity of the medium over this wide

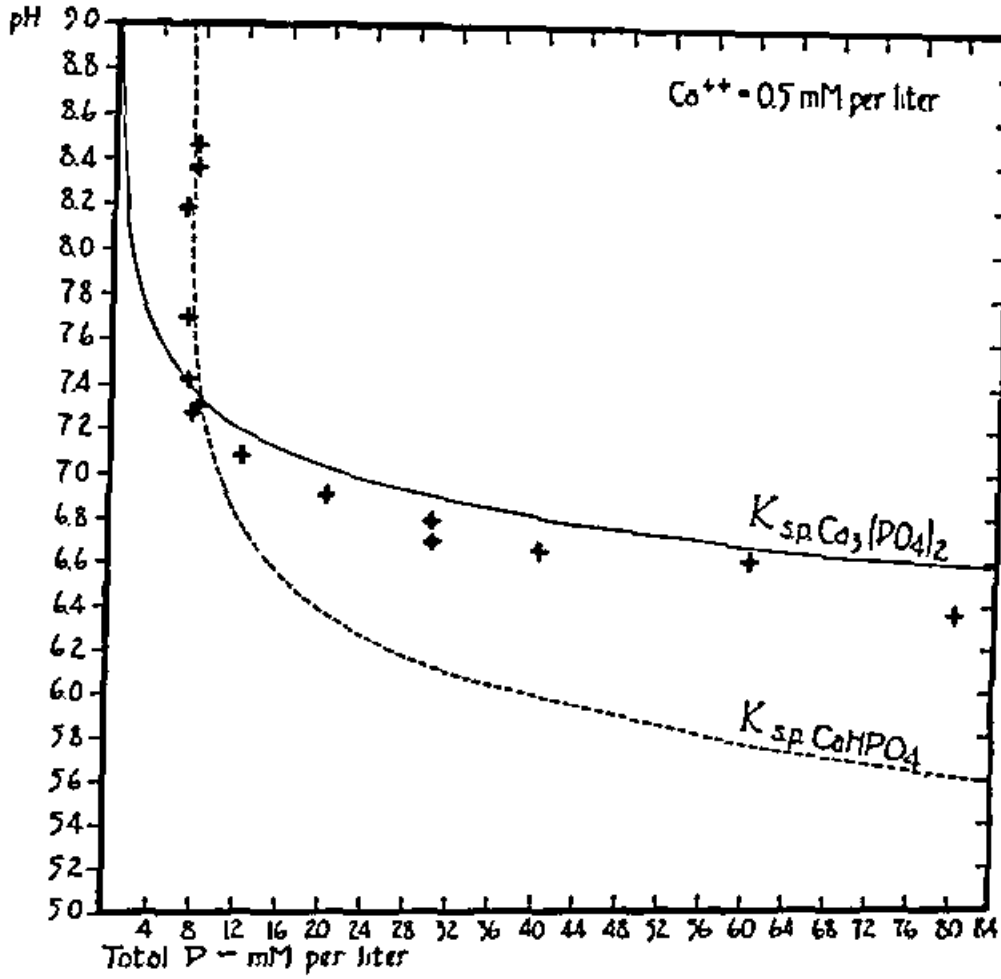

FIG. 14.—Relationship of calcium and phosphate concentrations and of ion products to calcification of rachitic cartilage in vitro. Total calcium (Ca^{++}) 0.5 mM/liter; pH and total phosphorus varied. Lines representing ion products drawn from $pK_{s\,p\,CaHPO_4} = 5.47$ (Shear and Kramer); $pK_{s\,p.Ca_3(PO_4)_2} = 23.1$ (Logan and Taylor); + indicates minimum ion products at which calcification occurred. Note that calcification above 7.3 is correlated with solubility product constant for $CaHPO_4$; below pH 7.3 with solubility product constant for $Ca_3(PO_4)_2$. (From Fig. 3, McLean, Lipton, Bloom, and Barron, Tr. Conf. Metab Aspects Convalescence, 14:33. Reproduced by courtesy of the publishers.)

range does not increase its ability to calcify rachitic cartilage matrix is conclusive evidence that the ion product of $Ca_3(PO_4)_2$ does *not play a decisive part in initiating and effecting calcification.* Such a change in hydrogen ion concentration increases the $PO_4^{=}$ concentration by a factor of 10, but increases the $HPO_4^{=}$ concentration by a factor of only 1.1. At any point in this range of hydrogen ion concentration small amounts of added phosphate will lead to calcification, indicating that the pH itself is not critical. These observations are not compatible with the belief that the $PO_4^{=}$ concentration, or any ion product incorporating it, is critical for calcification. They are consistent with the view that the ion product $[Ca^{++}] \times [HPO_4^{=}]$ is a determining factor.

Neuman and Neuman have shown that the serum of normal adults, while supersaturated or metastable with respect to the *mineral of bone, is undersaturated with respect to* $CaHPO_4$. Their view is that the solubility product constant for this salt defines an upper solubility limit for calcium and phosphate ions in solution. Deposition of the bone salt, by crystal growth, can and does occur at lower ion products; the *rate* of crystal growth depends upon collision frequency and is more rapid as the concentrations of the ions increase. The ion product is important in defining the conditions under which calcification will occur; it also has a direct bearing on the dynamics of the process. Moreover, since the proportions of Ca^{++} and of $HPO_4^{=}$ to the total Ca and total P are relatively constant, Howland and Kramer's empirical formulation, $Ca \times P$, is as reliable as is the ion product itself, or as the product of the activities of the ions.

The definition of an upper solubility limit for calcium and phosphate ions in the blood, without requiring that this limit be reached for deposition of the bone salt to occur, may explain a finding that has puzzled investigators for some years. It is well known that the phosphate concentration in the serum of adult man is about half that of the infant; yet new bone formation occurs in both. The higher ion product in the infant makes for more rapid calcification; the lower ion product in the adult does not exclude calcification, albeit at a lower rate. There is still no explanation for the observa-

tion that, while in the rachitic infant the phosphate concentration in the serum is at the level normal for the adult, calcification ceases in the infant while it continues in the adult.

CALCIFICATION: LOCAL MECHANISM

A complex mechanism, including both enzymatic and non-enzymatic factors, brings about calcification, under favorable humoral conditions, by transforming ions in solution in the fluids of the body to the crystalline state in calcifiable tissues.

What we call the *local mechanism* was first described by Robison in 1930, in recognition of the inadequacy of his phosphatase hypothesis as set forth in 1923. He characterized it as favoring deposition of the bone salt from supersaturated solutions. The mechanism was weakened, but not destroyed, by cyanide, by treatment of the hypertrophic cartilage with various solvents, or by desiccation. It was suggested that this mechanism is bound up with some labile structure of the tissue colloids but that it does not depend for its functioning on the living cell. Robison left the nature of the mechanism in an unsatisfactory state but adhered to the view that it is enzymatic in character. This conclusion was strengthened by observation of the inhibitory effects of iodoacetate and sodium fluoride on calcification of cartilage matrix *in vitro*. Robison stated, however, in 1934 that the conception of the local mechanism as an enzyme system did not preclude the possibility that factors of a different type, such as surface forces in the colloidal matrix, might also assist in the deposition of calcium salts.

At a later date, it was shown by others that cartilage matrix may retain the property of calcifiability *in vitro* after all enzymes have been inactivated, although there are quantitative differences in the response to the conditions in the solutions in which they are placed. These differences may be due either to the absence of enzymes or to the effects of the methods used to destroy the enzymes upon the non-enzymatic components of the local mechanism. The local mechanism, or portions of it sufficient to bring about characteristic calcification, will survive temperatures to 100° C., and will operate over a range in hydrogen ion concentration of at least pH 6.0–8.5, and is thus remarkably stable.

Further evidence of the stability of the local mechanism is derived from experiments in which calcification was produced *in vitro*, and the cartilage slices then decalcified in acetate buffer at pH 5.02–5.70. When again incubated in a calcifying solution, extensive calcification followed in every slice. Moreover, slices of cartilage aseptically removed and stored on moist gauze in a sterile Petri dish retained their ability to calcify for as long as ten days after removal from the animals.

In the present state of knowledge the property of calcifiability cannot be attributed to any single factor. Instead, it is necessary to postulate a local mechanism, including both physical and chemical components, all of which act together in the living organism to bring about calcification; the property which we describe as calcifiability is identical with that responsible for nucleation or crystal seeding.

NUCLEATION

Given a calcifiable tissue and humoral conditions favorable for calcification, something must occur to initiate the process. The chemist initiates crystallization of a salt by seeding a supersaturated solution. Precipitation and crystal formation follow, ordinarily to the point of equilibrium between the solid and the liquid phases. While there is much empirical information on how to produce nuclei, there is no satisfactory physicochemical theory of nucleation of a solid from a solution. We shall not attempt to deal with the nucleation of mineralization in bone and cartilage in physicochemical terms but will confine ourselves to a more general consideration of the available evidence.

First, it should be clear that two sets of conditions, of greater or lesser complexity, must be present in order to initiate calcification; we have called these the *humoral conditions* and the *local mechanism*. To define the humoral conditions more precisely, it may be said that the chief constituent ions of the bone mineral, calcium and phosphate, must be present in the medium from which mineral is to be deposited in such concentrations and such proportions as to create a condition of metastability; this is true whether nucleation *in vitro* or *in vivo* is under consideration. Such a condition of metastability, according to Neuman and Neuman, exists in the

blood plasma and is defined by them as a state of supersaturation with respect to the bone mineral, falling short of the degree of supersaturation required for spontaneous precipitation. A strictly quantitative description of this state, including its upper and lower limits, does not as yet exist.

Second, we have stated above that the property which we describe as calcifiability in a tissue is identical with that responsible for nucleation or crystal seeding; we have called this property, which has a high degree of complexity, the *local mechanism*. Much of the current effort in the study of the phenomenon of calcification is directed toward unraveling the essential features of this mechanism, whose primary function is that of nucleation of crystal formation. We are here concerned with the intimate details of the local mechanism, rather than with any further exploration of the property of metastability in a medium containing calcium and phosphate.

There is now general agreement that the collagen fibers of bone and cartilage have a dominant part in nucleation; the differences in the concepts advanced have to do mainly with the other factors concerned. The role of collagen is under intensive investigation by Glimcher and his collaborators, chiefly by *in vitro* studies. They regard collagen as a catalyst for the nucleation of apatite crystals and attribute this effect to the macromolecular aggregation state of the collagen fibrils. Experimentally, they have demonstrated that native type (640–700 A axial repeat) reconstituted collagen fibrils from normally uncalcified tissues, such as rat-tail tendon, calf skin, and fish swim bladder, are able to nucleate apatite crystals from metastable solutions of calcium and phosphate. Efforts to dissolve and reconstitute collagen from bone have not been successful. They propose the hypothesis that the juxtaposition of certain reactive groups in the collagen fibrils creates highly specific regions which act as sites for nucleation; their further studies are largely concerned with elucidation of the precise conditions required (Fig. 15).

The significance of these findings in relation to calcification of bone matrix *in vivo* is not entirely clear, even though the appearance of beginning mineralization *in vitro* is morphologically virtual-

ly identical with slightly mineralized collagen fibers from forming bone. Since the *in vitro* experiments have been performed with collagen from tissues that do not normally calcify, and since a very large proportion of the collagen in the animal body is not subject to calcification, it must be assumed that other factors, as yet not fully understood, are operative in differentiating calcifiable from non-calcifiable tissues. Glimcher suggests that mineralization, like

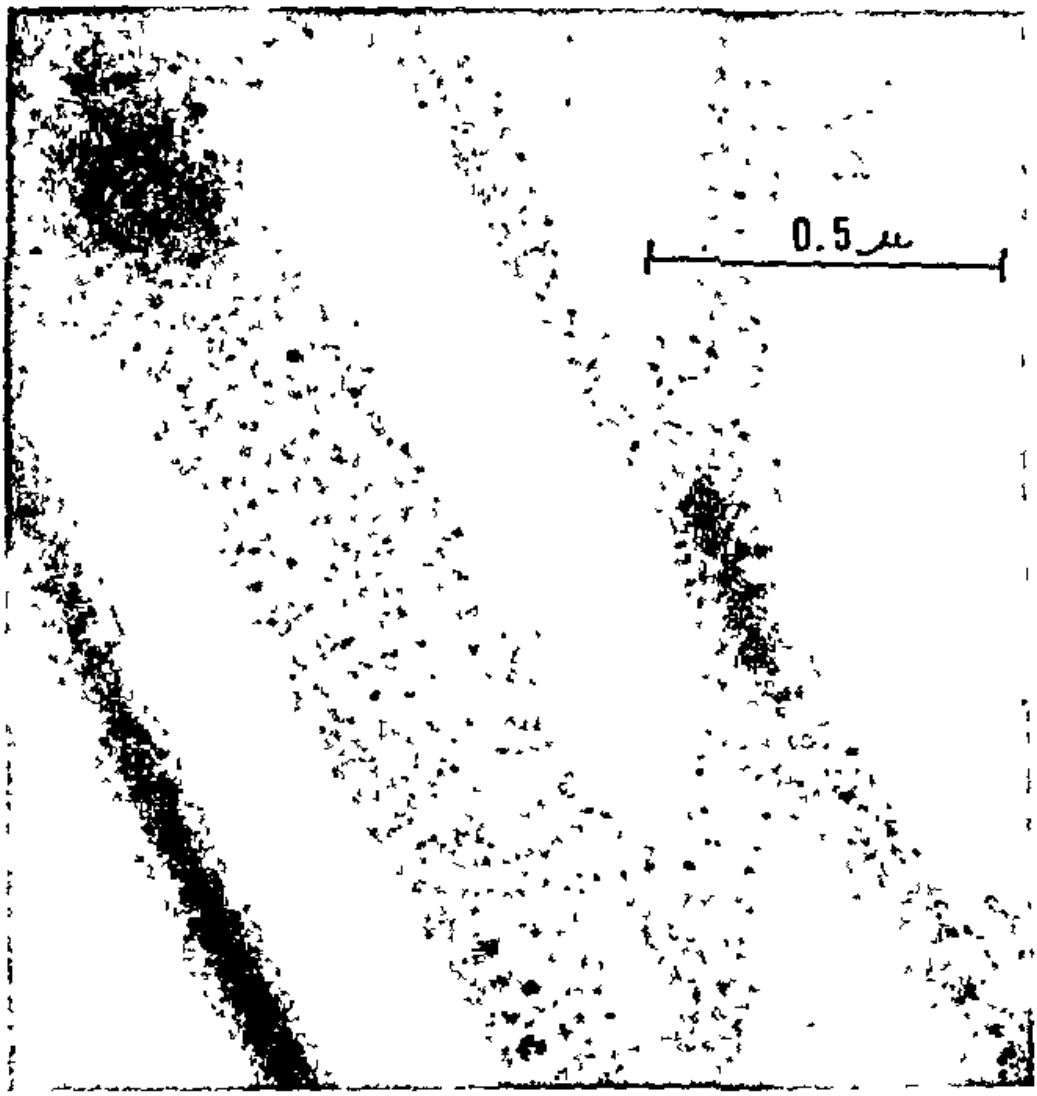

FIG. 15.—Unstained, unshadowed electron micrograph of an early stage of *in vitro* calcification of reconstituted collagen fibrils. Note periodic location of small, dense inorganic crystals, both longitudinally and laterally. ×66,600 (From original of Fig. 12, Glimcher, *in* Calcification in biological systems [Washington, D.C.: American Association for the Advancement of Science, 1960], p. 416. Reproduced by courtesy of the publisher.)

other functions of such vital importance to the organism, is under the control of many factors, delicately balanced to provide biologic and cellular regulation of the physicochemical mechanism that initiates crystallization.

The native collagen-rich tissues, such as rat-tail tendon and calf skin, which supplied much of the collagen used in the *in vitro* investigation of nucleation, failed to mineralize under conditions identical with those used in testing reconstituted collagen fibrils; these tissues did calcify after extraction of the components of the ground substance. From this, it has been inferred that the ground substance, *in vivo*, may exert a regulatory effect upon nucleation. At times it may shield the reactive sites in the collagen fibrils, and at times it may, by rapid depolymerization, release Ca^{++}, promoting nucleation. Thus the ground substance, as well as collagen, may well be implicated in nucleation.

Sobel and his collaborators have studied the possible role of the mucopolysaccharides of the ground substance in calcification. They accept the evidence that collagen induces nucleation but suggest that the complete system is more complex, and probably includes: (1) a specific form of acid-insoluble collagen; (2) sulfated mucopolysaccharide or mucoprotein; (3) enzyme systems such as glycolytic or citric acid cycles; (4) energy sources such as adenosine triphosphate or uridine triphosphate; and (5) a system concentrating calcium and phosphate ions. They visualize the active nucleating center as a site or adjacent sites, between the protofibrils of collagen, capturing a cluster of calcium and phosphate ions; the active center operates via a lock-and-key mechanism and is specific for conversion of the captured cluster of ions to a nucleus. These conclusions are based on experimental evidence obtained from *in vitro* studies.

Still another approach to a possible inhibitor of nucleation is that of Neuman and his collaborators. Disturbed by the lack of a function to be provided by alkaline phosphatase, Neuman suggested some years ago that the enzyme might function not to supply products but rather to destroy a substance inhibitory to crystallization. Fleisch and Neuman have recently reported that ultrafiltrates from dog, beef, and human serum do, in fact, interfere with

nucleation and that incubation of dog serum with phosphatase removed this inhibitory action.

It is apparent from the above that the phenomenon of nucleation of mineralization in bone and cartilage has not yet been fully and satisfactorily explained. It is encouraging to note, however, that much effort is being expended on this problem, with experienced investigators making use of the tools and concepts of modern physics and chemistry.

CRYSTAL GROWTH

With the widespread acceptance of the principle of nucleation of mineralization by collagen, with which are associated other, as yet ill-defined factors, there remain many details to be elaborated. We have considered what is known of nucleation, by which is meant formation of the initial fragments of the solid phase; following this, there is crystal growth until the crystals attain their full size.

A common misconception is to regard the formulation for hydroxyapatite as the formula of a molecule; and, with this, it is frequently assumed that formation of the solid phase requires simultaneous collision of the eighteen ions in the diagrammatic representation of a unit cell. The formulation of hydroxyapatite, or of the related bone mineral, however, represents only the *ratios* of all of the constituent ions of the solid phase in terms of smallest whole numbers; the unit cell describes only the arrangement of these ions in space. In the actual crystal both the the ions and the unit cells are repeated by the thousands or millions, in the spatial configuration characteristic for the particular cell; to imply formation of a unit cell as a fully formed unit of structure is an error in interpretation of the terms used.

Another misconception, related to this, is identification of precipitation with crystal formation. Precipitation implies formation of a solid phase from a solution, generally in aggregates of molecules, but without the orderly array of ions associated with crystal structure. Crystal formation, on the other hand, emphasizes the internal structure of the crystal; attainment of this structure occurs by step-wise addition of ions to the nucleus being transformed into the final form and size of the crystal. Formation of bone mineral is properly to be regarded as crystal formation, in close relation to an

organic matrix, rather than as simple precipitation within the matrix.

The internal structure of the crystals of bone mineral is determined by interionic forces, resulting in an array of ions characteristic for each particular mineral; it is not necessary to postulate a pattern or template which might pack each ion in its special location during the nucleation and subsequent growth of a crystal. *Orientation* of the crystals, with their long dimensions in the axis of the collagen fibers, with a co-oriented X-ray diffraction pattern of apatite and collagen, does, however, suggest a mechanism for the packing of the crystals. Such orientation is especially prominent in native bone but is not demonstrable when reconstituted collagen is calcified *in vitro*. Neuman has suggested that the orientation of hydroxyapatite crystals can be influenced by epitaxy, defined as crystalline intergrowth or oriented overgrowth, and he states that the weight of evidence is that collagen, itself a crystalline substance, can produce such an epitactic growth and orientation of the crystals. Observers are agreed that in the remineralization of demineralized bone the orientation of the crystals of mineral to collagen fibers resembles that in native bone.

CRYSTAL INTERIOR

The situation in the interior of the crystals of bone mineral is that the exact composition depends upon the conditions in the fluid from which they are derived, and that, once formed, substitutions and exchanges in the atomic structure are not readily brought about. Such substitutions and exchanges, however, do occur, and there is continuous, although slow, movement of ions from the exchangeable to the non-exchangeable fractions of the mineral. It has been shown, for example, that following administration of Ca^{45} to rats, the greater part of that retained in the skeleton is transferred, within a few weeks, to the non-exchangeable bone. For the movement of ions to the interiors of crystals, three mechanisms have been proposed: (1) recrystallization, in the classical sense, requiring dissolution and reformation of crystals, with incorporation of ions not previously within the crystal structure; (2) thermal diffusion or thermal vibration, analogous to the process by which

movement occurs within the structure of other solids; and (3) intracrystalline exchange, comparable to the exchange of surface ions but occurring at a much slower rate. It is assumed that, for complete or partial solution of crystals to occur, mediation of a biologic nature, such as by the local effect of an osteoclast, may intervene. Simple solution of apatite crystals in the fluids of the body, except perhaps for very thin or partial crystals on the surface, should be the exception rather than the rule.

SURFACE CHEMISTRY

The situation on the surfaces of the crystals is quite different from that in their interiors. Ca^{++}, $PO_4^{=}$, $CO_3^{=}$, and OH^{-} ions are exposed to the hydration shell and to the surrounding body fluids; ion exchange takes place readily, this being especially true in the more accessible and more reactive portions of the bone, i.e., the metabolic bone in the new and partially mineralized osteons. Here, the surfaces of the crystals are in contact with an aqueous medium in which they have a finite solubility. They are subject to constant ion exchange, as well as to other surface phenomena, and must be considered as a very dynamic system, with a labile structure. The surfaces are in equilibrium with their immediate environment; any attempt to describe them in static terms will lead to false impressions.

While this statement is generally acceptable with reference to the immediate environment of crystals, i.e., the extracellular fluid in direct contact with their surfaces, there are recurring suggestions that the blood plasma and even the bulk of extravascular fluids are not representative of equilibrium with the crystals of bone mineral. Instead, there is postulated a gradient, or even a physical barrier, which maintains a differential between the pH at the surface of the crystal and that in the circulating fluids; the nature of such a hypothetical barrier or gradient has not been elucidated. Nordin postulates a hydrogen ion concentration corresponding to pH 6.8 at the surface of the crystals; Neuman states that the pH in the immediate environment of the crystals must be lower than 6.8 and believes that the gradient between this and the hydrogen ion concentration in the circulating fluids in the body is maintained by the production

of acid by the cells of bone. Nichols also supports the concept that the concentrations of calcium and phosphate found in the extracellular fluid are the result of local pH changes at the bone mineral surface, determined by lactate formation.

The physiologic significance of such a pH gradient, from the surface of the bone mineral to the blood, would be that acid is essential for the solubilization of the mineral and for its movement from bone to blood. As will be seen later, in connection with regulation of this transfer by the parathyroid hormone and vitamin D, it is further believed that this regulation is mediated by the control of production of acid in the glycolytic cycle. In support of this thesis, there are quoted some observations which purport to show that the pH is low in areas of resorption of bone.

While we are prepared to accept the postulate that the *production* of acid in bone, and also production of the citrate ion, plays a part in the solubilization of the mineral, and hence its transfer from bone to blood, our view of the mechanism of this action is somewhat different from that of the authors just cited. We do not accept the idea that there is a pool of fluid, at pH 6.8 or below, surrounding the crystals in process of dissolution; in fact, we regard the concept of pH itself at the crystal surfaces as of doubtful validity. We are prepared to believe that hydrogen ions, liberated from the glycolytic cycle, may react with the crystal surfaces, as H^+ becomes available, in such a manner as to convert an insoluble salt of calcium and phosphate to soluble ions. Such a reaction, which we prefer to think of as occurring in a step-wise fashion, rather than in the presence of a pool of excess acid, might be represented as follows:

$$Ca_3(PO_4)_2 + 2H^+ \rightarrow 3Ca^{++} + 2HPO_4^{=}.$$

This question will be dealt with further under the topic of regulatory processes; here, the attempt is to visualize the dynamics of reactions at the crystal surfaces.

It may be assumed that the conditions at the surfaces of the crystals in reactive or labile bone, such as that localized in the new and still incompletely mineralized osteons, are different from those in the more stable and completely mineralized bone of the older

osteons and of lamellar bone. Our description of surface conditions applies primarily to reactive or metabolic bone; we assume that the surfaces of the crystals of stable bone mineral, while still capable of ion exchange, correspond to our description to a much lesser degree.

The special conditions at the surfaces of crystals include the effects of several forces, not mutually exclusive. The classical adsorption phenomena, in which the bonding is primarily the result of van der Waals forces and in which the adsorbed material may be present in multilayers, refer primarily to the adsorption of gases by solids; ions in solution are not known to undergo classical adsorption. Chemisorption refers to electrovalent or covalent bonding, in which the chemisorbed material is usually limited to a surface monolayer. Ions in solution are frequently chemisorbed; an excellent example is the adsorption of calcium and phosphate ions from serum shaken with lead phosphate; hydroxyapatite is also an effective chemisorber for these ions. Many attempts have been made to explain the uptake or exchange of ions by the bone mineral, in contact with fluids of the body, in terms of chemisorption. Currently, however, the emphasis is on exchange and transfer of ions between the body fluids and crystal surfaces. This concept has been subjected to careful quantitative studies, which have led to elucidation of stoichiometric relationships and to the conclusion that the dynamics of the behavior of ions at the crystal surfaces are best expressed in terms of ion exchange, without, however, eliminating the possibility of chemisorptive forces acting concurrently.

ION TRANSFER AND ION EXCHANGE

Strictly speaking, the term ion exchange implies a one-for-one exchange of ions between a solid and the fluid which bathes it. This exchange may be *isoionic*, i.e., the exchange of one ion for another *of the same element, or heteroionic, exchange for a different ion.* The rapid physiologic exchange between the bone mineral and the fluids of the body is almost entirely isoionic.

A more general term is ion transfer, which designates the movement of ions between bone and blood, without requiring an equal number in exchange. It is assumed that there is a pool of labile mineral, in the reactive or exchangeable bone, which can move

rapidly into the circulating fluids when they are depleted; conversely, an excess of calcium in the blood is transferred rapidly to the labile pool.

This concept of labile calcium, and of ion transfer, which does not require any destruction of organic matrix, bears a superficial resemblance to the older concept of *halisteresis*, which described a leaching of mineral out of bone, and may seem to contradict the assertion, now commonly made, that resorption of bone destroys mineral and matrix together. Halisteresis, however, was a term used to describe an older concept of rickets, which was that in the pathogenesis of rickets, mineral was dissolved from bone, leaving the organic matrix intact. This concept was disposed of by Pommer, in 1885, who first showed that in both rickets and osteomalacia there is a failure of organic matrix to calcify, resulting in the characteristic uncalcified osteoid tissue. The concept of labile calcium is simply that a portion of the calcium, variously designated as labile, exchangeable, metabolic, or reactive, is capable of moving in or out of bone, in response to a dysequilibrium with the immediate environment, without any necessary disturbance of the organic matrix with which it may be associated. This, then, draws a sharp distinction between resorption and ion transfer; they occur in different places, under different conditions, and by different mechanisms.

For reactive bone, there is conceptually a choice of four positions in which a particular ion may be at any one time and still be in relation to hydroxyapatite. It may be in the interior of a crystal and, hence, in a relatively stable position. Or it may be in a special relation to the surface, in one of three positions: (1) in the crystal surface; (2) adsorbed on the surface of the crystal, by chemisorptive forces; or (3) in solution in the hydration shell. Where the ions of hydroxyapatite or carbonate hydroxyapatite are concerned, Ca^{++}, $PO_4^{=}$, OH^{-}, and $CO_3^{=}$, the distinctions at the surface are rather arbitrary. The system is in a dynamic state; the surface undergoes continual exchange; and its extension into the aqueous phase is blurred dimensionally and energetically. It is assumed that the degree to which this is true depends upon the maturity of the crystal and

upon its environment; in no case, however, is it probable that mineral in bone exists in the state of stability of a dry crystal in contact with air.

DIFFUSE ACTIVITY AND LONG-TERM EXCHANGE

When Ca^{45} or Ra^{226} is taken up by bone, activity appears not only in exchangeable or reactive bone, undergoing appositional growth, but also in pre-existing bone, producing a *diffuse* and relatively uniform low level of darkening in an autoradiogram. This diffuse deposition, believed to represent a one-for-one exchange of ions, isoionic or heteroionic, with those present in compact bone, has been designated as *long-term exchange*, defined as an exchange process with time constants much greater than a week. While a radioisotope taken up in this manner does not produce as striking a picture in autoradiograms as do radioelements taken up in newly forming bone, where strong images are produced, it is estimated that as much as 50 per cent of the total uptake of Ca^{45} or Ra^{226} may be in the form of the *diffuse component.*

The justification for referring to this phenomenon, regarded by some as an irreversible uptake, as long-term exchange is that observations of Ca^{45} transfer indicate that a significant fraction of the total calcium turnover in the skeleton occurs as a result of such exchange, and that this may play an important part in the regulation of the serum calcium level. Evidence is accumulating that Ra^{226} is removed slowly from the mineral, without the necessity of direct resorption, and apparently by the same exchange process that deposited it originally.

Localization of the diffuse component, in relation to the crystals of bone mineral, remains uncertain. It is assumed that the first deposition of the radioisotope is at the surfaces of crystals, in exchange for an equal number of Ca^{++} ions. Beyond this, it is a reasonable assumption, in the present state of knowledge in solid-state physics, that any ions so deposited that are capable of substituting for Ca^{++} in the crystal structure may, in time, find their way into the interior of the crystal. Whether this is the case for Ra^{226}, which may be demonstrated in compact bone many years after the original uptake, has not been shown.

CHAPTER VII

Enzymes and Bone

Most of the attention to the enzyme systems in cartilage and in bone has been directed to their possible role in calcification. Many of the enzymes in these tissues, however, as in the soft tissues, have primary functions with relation to the metabolic activities of the cells. There is no evidence that any enzyme system is required for calcification of the matrix of bone, as there is for cartilage matrix. The richness of osteoblasts in phosphatase has suggested, however, that the presence of this enzyme is involved in the formation and deposition of the bone mineral. That the bone matrix itself is free from phosphatase throws doubt on this interpretation; phosphatase is present in the matrix of cartilage in the zone of provisional calcification

Interest has been centered on the possible role of alkaline phosphatase, phosphorylase, and the enzymes of the glycolytic cycle in calcification of the matrix of hypertrophic cartilage during endochondral formation and growth of bone. Here the evidence for participation of enzymes in the calcification process is more direct, although any conclusions must still be subject to reservation.

Calcification of cartilage matrix may be produced *in vitro* after all enzymes have been inactivated. Inactivation of the enzymes, to the extent that no phosphatase activity is demonstrable, may be accomplished by treatment with silver nitrate, 0.6×10^{-2} M, or bichloride of mercury, 10^{-2} M, or by heating to temperatures between 60° and 100° C. Such inactivation, of course, affects the enzymes both in the matrix and in the cells; following inactivation the conditions in bone matrix and in cartilage matrix are comparable; both are free from enzymes. Even after these procedures, characteristic calcification of cartilage matrix may be produced if the phosphate con-

centration in the medium is sufficiently high. These observations suggest that phosphatase may play an important part in the preparation of the matrix for calcification; after the property of calcifiability has been established, the presence of the enzyme is no longer necessary.

Our examination into the enzymes of bone and cartilage will not be restricted to the part they may play in calcification. We shall attempt, insofar as the evidence permits, to review other functions of enzymes found in association with these tissues.

ALKALINE PHOSPHATASE

In 1923 Robison announced that he had found an enzyme in the ossifying cartilage of young rats and rabbits which rapidly hydrolyzes hexosemonophosphoric acid, yielding orthophosphoric acid He suggested that this reaction, by adding to the local concentration of phosphate ions, might be a factor in bone formation. From this discovery it was an easy step to the assumption that the action of the enzyme is critical in calcification. Indeed, the discoverer of phosphatase and his followers went beyond the evidence, and a certain amount of retraction was inevitable. This was followed by a restatement of the case for phosphatase, to include a second mechanism; the current literature is still markedly influenced by the early and clearly untenable hypothesis.

The original theory was based not only on the discovery of alkaline phosphatase in bone but also on the demonstration that rachitic cartilage matrix will calcify *in vitro* when phosphate is furnished in the calcifying medium only in the form of phosphoric esters. The difficulties encountered and recognized by the originator of the theory were: (1) that the optimum pH for the action of the enzyme is high, namely, 9.4; (2) that the concentration of phosphoric esters in the fluids of the body, suitable as substrates for the enzyme action, is low, amounting only to about 0.5 mg. of phosphorus per 100 ml. of fluid; and (3) that other tissues, which do not calcify, are rich in an identical or similar enzyme. The literature of the last thirty-five years includes many attempts to reconcile these

difficulties with the possibility that alkaline phosphatase has an important role in calcification.

Bone phosphatase, in addition to its localization in bone, is characterized by its hydrolysis of monoesters of phosphoric acid and its optimal activity at pH 9.4. It shares these qualities with similar enzymes from other sources, of which the best studied are those from blood plasma and from intestinal mucosa. Some rather subtle differences, mainly in the resistance to inhibitors and in the optimum pH, have been reported between phosphatases from different sources. Since distribution studies have failed to reveal significant differences between phosphatases from mammalian tissues, it will suffice, for our present purposes, to consider the alkaline phosphatase from bone as non-specific and to treat it in terms of its activity.

By keeping the incubation time short to minimize diffusion artifacts, and to demonstrate sites of activity preferentially, Pritchard, in a study of phosphatase in rat femurs, found that areas of mesenchyme in which centers of ossification later appeared showed that there was activity before osteoblasts had differentiated. The enzyme first appeared in the nuclei of mesenchymal cells; as the cells enlarged and assumed the form of osteoblasts, the activity became more intense and appeared in both nucleus and cytoplasm, and also extracellularly in collagenous fibers. With the appearance of definitive osteoblasts, however, enzyme activity fell abruptly, especially in the nucleus. The organic matrix being deposited between and around the osteoblasts gave no reaction for phosphatase. Resting osteoblasts and osteocytes showed little or no activity. Analogous changes were observed in cartilage preceding its replacement by bone in endochondral ossification. As the cartilage cells began to hypertrophy, enzyme activity appeared in the nucleus first, then in the cytoplasm, and then extracellularly. Soon after the appearance of the enzyme in the cartilage matrix calcification began. As the cells became hypertrophic and began to degenerate enzyme activity disappeared.

Except for the localization of phosphatase in the cartilage matrix, its distribution in the cellular elements of bone and carti-

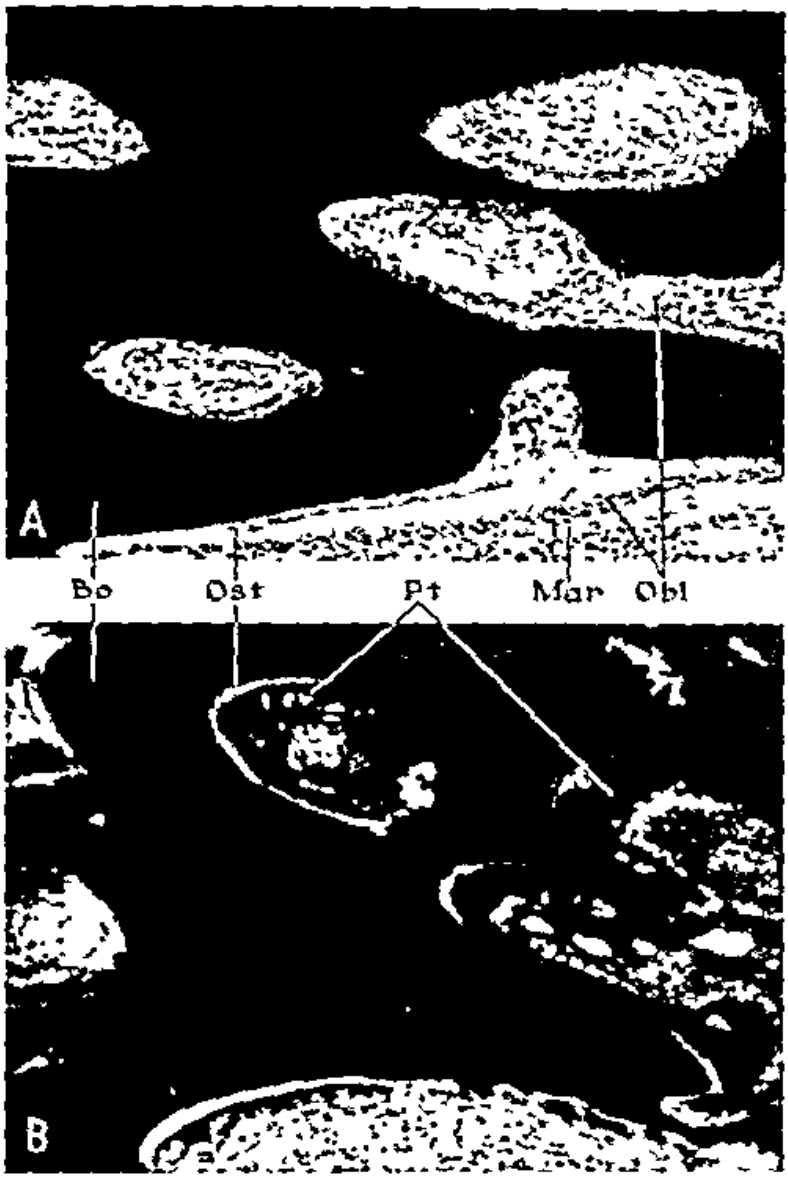

FIG. 16.—Sections illustrating distribution of alkaline phosphatase in bones of a puppy with minimal rickets *A*, photomicrograph of an undecalcified longitudinal section through the shaft of a rib of a puppy fed control diet without vitamin D; *bo*, indicates calcified bone; *ost*, borders of uncalcified osteoid tissue, *obl*, osteoblasts; *mar*, bone marrow. *B*, similar area of a section from the same block incubated with sodium glycerophosphate in presence of calcium before staining *Pt* indicates heavy deposits in layers of osteoblasts, showing localization of phosphatase; other symbols as in *A*. Note borders of uncalcified osteoid tissue free from phosphatase Alcohol fixation; silver nitrate–hematoxylin-eosin. ×103. (From original photomicrographs of Fig. 7, Freeman and McLean, Arch. Path., 32:401. Reproduced by courtesy of the publishers.)

lage, including the cytoplasm and the nucleus, does not suggest any direct relationship to the deposition of the bone salt. Especially striking is its absence from calcifiable bone matrix, as in rickets, at a time when the overlying osteoblasts are rich in the enzyme (Fig. 16). It is of considerable interest that the distribution of a mucopolysaccharide, as demonstrated by histochemical means, coincides with sites rich in alkaline phosphatase.

The literature on phosphatase in the living organism is chiefly concerned with the hydrolysis of phosphoric esters. The enzyme can also serve as a catalyst in the synthesis of these esters, and it *may* have such an action *in vivo*. Attention has been called to the high acid phosphatase content of osteoclasts and to the possibility that the enzyme plays a part in resorption.

Although phosphatase hydrolyzes and synthesizes the monoesters of phosphoric acid, this by no means limits the functions of the enzyme to this activity. There is abundant evidence for the view that the enzyme plays an important part in the manufacture of a fibrous protein. This conclusion is based partly on an increase in cytoplasmic basophilia of the osteoblast, concomitant with an increase in phosphatase; on an increase in the size of the nucleolus; and on a simultaneous increase in the intercellular matrix, particularly of its collagenous component. Moreover, it is believed that nuclear phosphatase activity may be concerned with the intrinsic metabolism of cell differentiation. That there is an intimate relationship in distribution between phosphatase and mucopolysaccharides suggests that the enzyme may also play a part in the formation of the ground substance of bone. Since the property of *calcifiability* appears to reside in the organic matrix, this relationship is consistent with the view that the conferring of this property on the matrix may be a function of phosphatase.

SECOND MECHANISM

In 1930 Robison postulated a dual mechanism in calcifying hypertrophic cartilage. The two parts were: (1) the phosphatase mechanism, which produces supersaturation with respect to the bone phosphate, and (2) the second mechanism, which favors the

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Robison continued to regard this second mechanism as enzymatic in nature; and in 1936 he stated that the two mechanisms, which had been empirically distinguished, might form one complex enzyme system by which phosphoric esters are synthesized from inorganic phosphate and subsequently hydrolyzed to liberate phosphate ions for the precipitation of the bone salt. The case for the dual mechanism was supported by demonstration of the simultaneous presence of glycogen and phosphatase in the cells of cartilage of which the matrix was ready for calcification. It was suggested that the senescent or hypertrophic cartilage cells provide both the phosphatase enzyme and the glycogen, the glycogen on hydrolysis yielding hexosephosphoric acid esters which, under the action of the phosphatase and the calcium of the body fluids, lead to the deposition of an insoluble calcium phosphate in the matrix.

There remains another possible role for phosphatase in the second mechanism. Neuman and his co-workers have suggested that alkaline phosphatase acts locally to destroy an organic phosphate, believed to inhibit the nucleation of crystals of the bone mineral. While this proposal has some experimental support, it requires further investigation. Henrichsen has agreed with Robison that phosphatase is essential for the second mechanism and regards release of phosphatase from cells, by death of the cells or otherwise, as critical for nucleation.

PHOSPHORYLASE

Beginning in the cartilage models of bone in the embryo and continuing into postfetal life as long as growth of bone by endochondral ossification persists, an enzyme system for glycolysis, similar to that in muscle, is present in the cartilage. The appearance of this system coincides with the hypertrophy, the degenerative changes, and the presence of glycogen observed in cartilage cells preparatory to their disintegration and the calcification of the matrix. No similar system has been described in bone tissue.

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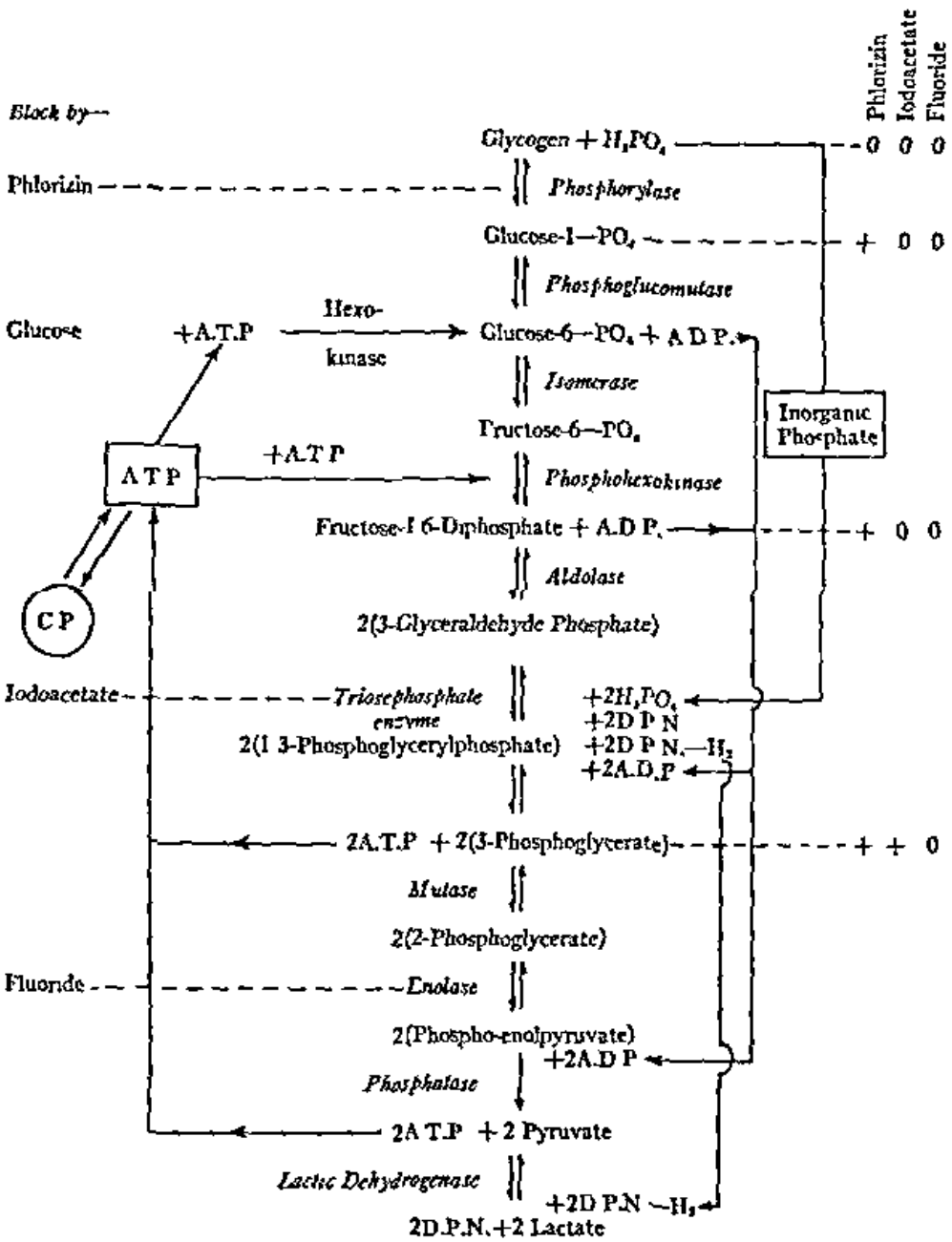
glycogen and phosphatase in regions in cartilage where calcification is about to occur. An important link in this chain was the demonstration, in 1941, of a phosphorylase in calcifying cartilage. This enzyme initiates phosphorylative glycogenolysis by catalyzing the breakdown of glycogen and the formation of glucose-1-phosphate. Upon its discovery in calcifying cartilage it was believed to lead to synthesis of potential substrates for phosphatase in zones of calcification before blood sources become available, and to supplement these blood sources thereafter.

GLYCOLYTIC CYCLE

Figure 17 illustrates the glycolytic cycle, beginning with the phosphorylation of glycogen to form glucose-1-phosphate and continuing down to the formation of lactate. Not all the steps illustrated in this scheme have been verified for calcifying cartilage; in addition to phosphorylase and phosphatase, the following enzyme activities have been identified: phosphohexoisomerase, phosphohexokinase, hexokinase, aldolase, α-glycerophosphate dehydrogenase, triosephosphate dehydrogenase, enolase, and lactic dehydrogenase. The presence of the following phosphorylated intermediates has also been established: glucose-1-phosphate, *fructose-6-phosphate*, *hexose diphosphate*, *phosphoglyceric acid*, inorganic orthophosphate, phosphopyruvic acid, and adenosine triphosphate. These are sufficient to lead to the conclusion that the glycolytic systems of muscle and of calcifying cartilage are very similar, if not identical.

The glycolytic cycle has been implicated in the calcification of cartilage matrix, but not of bone. As illustrated in Figure 17, phlorizin, iodoacetate, and fluoride inhibit glycolysis, but at different points in the cycle. Thus phlorizin inhibits phosphorylase and, consequently, phosphorylative glycogenolysis; iodoacetate inhibits the triosephosphate enzyme; the fluoride inhibits enolase. Correspondingly, each of these inhibitors blocks calcification when phosphorus is supplied as inorganic phosphate or as any of the phosphoric esters above the location of an inhibition in the cycle.

An attempt has been made to show that the local mechanism in



A.T.P. = Adenosine triphosphate *A.D.P.* = Adenosine diphosphate
D.P.N. = Diphosphopyridine nucleotide (Coenzyme I) *C.P.* = Creatine phosphate

FIG 17.—Diagram illustrating effect of inhibition of enzyme activities upon calcification of rachitic cartilage *in vitro*. Inhibition of enzymes participating in glycolytic cycle by phlorizin, iodoacetate, or fluoride interferes with calcification when phosphate is supplied in the form of a compound above the reaction for which the inhibited enzyme is required. The glycolytic cycle illustrated is that from muscle. (Adapted from Duncan, *Diseases of metabolism* [2d ed.; Philadelphia: W. B. Saunders Co., 1947], Fig 10, p. 56. Reproduced by courtesy of the publishers.)

calcification is dependent upon anaerobic glycolysis in the tissue; this attempt has failed. Anaerobic glycolysis may be inhibited *in vitro*, to the extent of 70–90 per cent, by iodoacetate or fluoride; this inhibition may be accompanied by a reduction in the amount of calcification, but this reduction is easily overcome by adding slightly more phosphate. Moreover, glycolysis, in the absence of inhibiting agents, proceeds at the same rate, whether or not the incubating medium contains enough phosphate to induce calcification.

Even after making allowance for the facts that the inhibitions and blocks mentioned are only partial and that for the most part they can be by-passed by providing enough of the substrate or of inorganic phosphate, this scheme and its experimental validation are impressive evidence for the participation of the glycolytic enzyme system in the calcification process. It has the fault that the cycle, as now understood, does not lead to a phosphoric ester to be acted upon by alkaline phosphatase; consequently, the scheme does not clarify the role, if any, of phosphatase in the deposition of the bone mineral. One must conclude that all efforts to implicate phosphatase in calcification have failed, in spite of a most promising and apparently self-evident start a generation ago. There is, however, current interest in adenosine triphosphate (ATP), also an important link in the glycolytic cycle (see Fig. 17). ATP serves many functions in metabolism, and is now believed to serve as a phosphate donor in calcification. It is pointed out that ATP is the only acid-soluble ester that accumulates as a result of glycogenolysis in ossifying cartilage. The interest in ATP is also strengthened by the demonstration of *pyrophosphate* as a constituent of normal bone. Since inorganic pyrophosphate is not an effective calcifying agent, it has been suggested that the role of ATP is that of transferring pyrophosphate to some component of the organic matrix by a transference mechanism; the working hypothesis is that a *transphosphorylation* reaction takes place in ossifying bone according to the scheme:

$$\text{Collagen–NH}_2 + \text{ATP} \xrightarrow{\text{ATPase}} \text{Collagen–NH–P}\sim\text{P} + \text{AMP}.$$

Linked with this proposal is the belief that the glycolytic cycle is important for ossification only for its ability to form pyruvic acid, by the oxidation of which the cartilage cell may synthesize ATP. Because of the role of insulin in the multienzyme system which controls the metabolism of ATP and the biochemical reactions dependent upon it, e.g., biosynthesis of chondroitin sulfate, attention is currently being directed to the influence of insulin upon calcification and ossification.

Granted that the glycolytic cycle may play a part in the calcification of certain tissues, the mechanism of its participation still remains in doubt. The transport of an original phosphate ion through the cycle, with its reappearance as inorganic phosphate, does not result in an increase in the number of such ions. The mechanism might be needed to transfer phosphate ions across cell membranes or to move them against concentration gradients. The high-energy phosphate bonds of some of the esters of the glycolytic series might serve a purpose useful in calcification. Or it is conceivable that cyclic accumulation and release of phosphate ions might occur, with temporary local increases in their concentration. Such a storage mechanism is implicit in some of the early theories of the action of alkaline phosphatase; it still has not been shown to exist, either for phosphatase activity or for the glycolytic cycle.

ACID PHOSPHATASE

A phosphatase with optimum activity in acid solutions, pH 6.0, found in the prostate gland and attaining high levels in the plasma in patients with prostatic cancer, has, until recently, not been reported to have a specific relation to bone. Current reports are to the effect that the calcifying matrix of bone and dentin exhibits a genuine acid phosphatase activity, which is abolished or diminished by procedures known to inactivate or inhibit the enzyme. It is also reported that, while osteoblasts and osteocytes exhibit alkaline phosphatase activity, this is not found in osteoclasts; a high acid phosphatase activity has been found in these cells. The role of acid phosphatase in bone is even more obscure than that of alkaline phosphatase. Present concepts are that the alkaline phos-

phatase activity is an index of osteoblastic activity, while acid phosphatase activity in bone reflects an osteolytic process.

OTHER ENZYMES

Since bone is a living tissue and since many enzyme systems are common to a wide variety of cells, it may be assumed that the cellular elements of bone have their share of such enzymes. Little is known about bone in this respect. Cartilage is generally deficient in respiratory and oxidative enzymes. This is consistent with the relative avascularity and very low oxygen tensions prevailing in cartilage. The oxygen uptake of cartilage homogenate is small, though measurable.

The role of enzymes in the resorption of bone has not been clarified. It is probable that the collagen of bone is dissolved by enzymatic action and that the mucopolysaccharides of the ground substance are made soluble by depolymerization. In neither case is information available for complete elucidation of the sequence of events in resorption.

It has long been assumed that a proteolytic enzyme must be active in the destruction of collagen. The osteoclast is in a position to provide such an enzyme, but there is no evidence that the elaboration of a proteolytic enzyme by osteoclasts is responsible for the solution of the proteins of the bone matrix. The enzyme hyaluronidase acts to depolymerize hyaluronic acid. This mucopolysaccharide has been identified in bone; the enzyme acts similarly on chondroitin sulfate; its role in resorption can only be surmised; hyaluronidase has not been demonstrated in osteoclasts.

Histochemical demonstration of succinic dehydrogenase activity in osteoclasts has been interpreted as presumptive evidence that the tricarboxylic acid cycle is involved in resorption. Other enzymes found in osteoclasts, including β-glucuronidase and aminopeptidase, appear to confirm the hypothesis that these cells have high metabolic activity. Van Reen has made estimations of the activities of aconitase and isocitric dehydrogenase in various areas of the femurs from rabbits and dogs but has not investigated their distribution at the cellular level.

Tessari has shown an increase in glutamic-oxalacetic transaminase activity (GOT) in the areas of spongy bone subject to the resorptive activity of growth and has also found that a similar increase occurs following administration of parathyroid extract. The role of transaminase activity in resorption remains to be clarified.

As a part of the mechanism presumed to be involved in the transfer of phosphate from adenosine triphosphate, Cartier and Picard have demonstrated the presence of a non-extractable enzyme, ATP-ase, which differs from alkaline phosphatase in histologic distribution and has an optimum pH of 8.0. It is activated by magnesium ions

Other enzymes reported to exist in bone, and under investigation with reference to their physiologic significance, include esterases, proteases, cytochrome oxidase, 5-nucleotidase, and β-glucosidase. Of the enzymes of the citric acid cycle, those studied in bone include citrogenase, aconitase, isocitric dehydrogenase, and malic dehydrogenase. Doubtless there are many more enzymes in bone, and their activities remain a fruitful field for inquiry.

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CHAPTER VIII

Resorption of Bone

Resorption refers to the destruction or solution of the elements of bone. Our knowledge of the morphologic aspects of this process begins with a monograph by Koelliker, one of the great early histologists, who wrote a clear description nearly ninety years ago, still classic in the subject. Koelliker not only described resorption, with its role in the growth and reconstruction of bone, but also described and named the osteoclast. He found that bone mineral and bone matrix are absorbed simultaneously, a fact repeatedly rediscovered in modern times; and he offered speculations concerning the mechanism of resorption to which relatively little has been added to this day. In many respects resorption is no more clearly understood than it was when Koelliker left it; moreover, much effort has had to be expended in clearing away misconceptions introduced by others attempting to improve upon his work.

Resorption is, in essence, the putting into solution of a complicated structure, in such a fashion that it disappears, its end-products entering the blood stream. Of the components of bone, a small fraction is already in fluid form and hence easily disposed of; the remainder is in solid form, for the most part insoluble or soluble with great difficulty in the aqueous fluids. This is to say that, in order to resorb bone, it is necessary that it be reduced to substances soluble in water and that these be transferred to the fluids of the body.

COMPONENTS OF BONE

Of the solid components of bone, three substances together make up the main bulk. These are: (1) the bone mineral; (2) a fibrillar protein, collagen; and (3) the ground substance, characterized by its content of one or more polysaccharides. The ground substance is

closely related to the interstitial fluid, and its mucopolysaccharides may be made soluble in water by a change in their state of polymerization. Since no enzyme specific for the depolymerization of chondroitin sulfate has been isolated, hyaluronidase is described as mediating this process.

Collagen can be rendered soluble rather readily. At the temperature and hydrogen ion concentration of the fluids of the body it can be dissolved by digesting or disintegrating its protein structure. This can be accomplished in the test tube by proteolytic enzymes, including collagenase.

Koelliker, in 1873, concluded that the osteoclast erodes bone by chemical means, without further specifying the nature of the chemical action required. Later, others added the assumption that the action is a combination of that of an acid with that of a proteolytic ferment; the necessity for the presence of acid to account for the solution of the bone mineral has been a difficult stumbling block in the formulation of a theory of resorption. This situation has been changed by the introduction of a group of compounds, known as chelating agents, of which the prototype is ethylenediamine tetraacetic acid (EDTA).

CHELATING AGENTS

Chelating agents are characterized by the formation of very poorly dissociated complexes with metallic ions. Together with this property, which involves the formation of ring structures with co-ordination linkages, chelate structures exhibit greatly increased stability, lowered solubility in water, and changes in the dielectric constant. These characteristics differentiate chelate structures from such complex ions as are formed by calcium with citrate; from the physiologic point of view the important difference is that the ability of a chelated complex to ionize in solutions is very much below that of calcium citrate.

It is now a common laboratory procedure to decalcify bones and teeth with chelating agents for histologic purposes; this is possible even in neutral or strongly alkaline solutions. It may be safely assumed that the bone salt may be dissolved whenever another sub-

stance with a stronger affinity for calcium is in a solution in contact with bone. This removes one of the obstacles to forming a hypothesis for the resorption of bone. Such a hypothesis may now account for the solution of bone salt, collagen, and ground substance, all at the hydrogen ion concentration of the body fluids.

For a long time it has been agreed that both the organic and the inorganic components of bone are resorbed together, leaving smooth surfaces at the loci of resorption. If it be assumed that there is continuously applied to the surface of bone a solution which will depolymerize mucopolysaccharides, digest collagen, and hold calcium in a firm and soluble combination, this constitutes the basis for a working hypothesis for the mechanism of the resorption of bone. Such a mechanism would require only certain enzyme systems and an organic substance to combine with calcium. This does not conflict with any known facts, but there is no positive evidence that it adequately describes the process. Nor is it suggested that any known chelating agent is present in bone; the possibility is that substances having similar properties may be formed in the organism.

ROLE OF OSTEOCLAST

It can no longer be doubted that osteoclasts play an active part in the resorption of bone. Whereas for many years the evidence to this effect was indirect, and dependent upon the study of fixed tissues, the studies of Gaillard, of Goldhaber, and of Hancox, all of whom have observed resorption of bone in tissue culture, making use of time-lapse motion pictures, have conclusively demonstrated the melting away of bone before advancing osteoclasts, which display very active ruffled borders and energetic *pinocytosis*. The ruffled border as seen in tissue culture appears to correspond to the brush border or striated border, between the osteoclast and the underlying surface of bone in fixed preparations, which has long been a subject of controversy.

The observations of living osteoclasts have also shed light upon the mechanism of osteoclastic resorption of bone. They leave little d[illegible] these cells have a secretory activity, and that this serves to [illegible] both the mineral and the organic matrix of

bone. They do not, so far at least, give a final answer to the problem of the possible phagocytic activity of the osteoclasts. Their secretory activity results in the liberation of debris; some of this may be swept up by the undulating ruffled borders of the osteoclasts, and ingested and dissolved by these cells; such a mechanism is suggested by a combination of the undulating ruffled border and the amoeboid movements of the entire cells, accompanied by pinocytosis. On the other hand, at least in tissue culture, there are large numbers of very active macrophages, seemingly adequate for phagocytosis of such debris as is present. Moreover, if the osteoclasts secrete a substance or substances capable of chelating calcium and solubilizing the collagenous fibers of the matrix, as we have suggested, it is not necessary to assume that all of the loosened or fragmented material liberated from the disappearing bone needs to be dealt with by ingestion into cells.

PHAGOCYTOSIS

The above calls for a further review of the evidence for and against the concept of phagocytosis, by the osteoclasts themselves, as at least a part of the mechanism of osteoclastic resorption of bone. Such a suggestion has appeared in the older literature, reviewed by McLean and Bloom in 1941. They studied the mobilization of bone salt in a variety of animals under the influence of toxic doses of parathyroid extract, staining undecalcified sections of fixed bone tissue by the von Kóssa method, for visualization of the mobilized mineral. They found aggregation of mineral, particulate during the life of the animal, in the macrophages and megakaryocytes of the bone marrow but found also that the osteoclasts were singularly free of these aggregates. They concluded that osteoclasts do not participate in the phagocytosis of the mineral liberated from resorbing bone (Fig. 18).

Subsequent observations, by the use of newer methods, require a reopening of this question. Scott and Pease, by use of the electron microscope, have found rodlets of bone mineral lying within the cytoplasm of osteoclasts in untreated animals and have interpreted this finding as phagocytosis of the crystallites (Fig. 19). Arnold and Jee, studying the alpha tracks of plutonium in autoradiograms, have

demonstrated that this material is concentrated in osteoclasts; they interpret this as indicating osteoclastic phagocytosis of this bone-seeking element. Thus the electron microscopic and the autoradiographic evidence on this subject is in conflict with the earlier findings with the light microscope; further clarification is required.

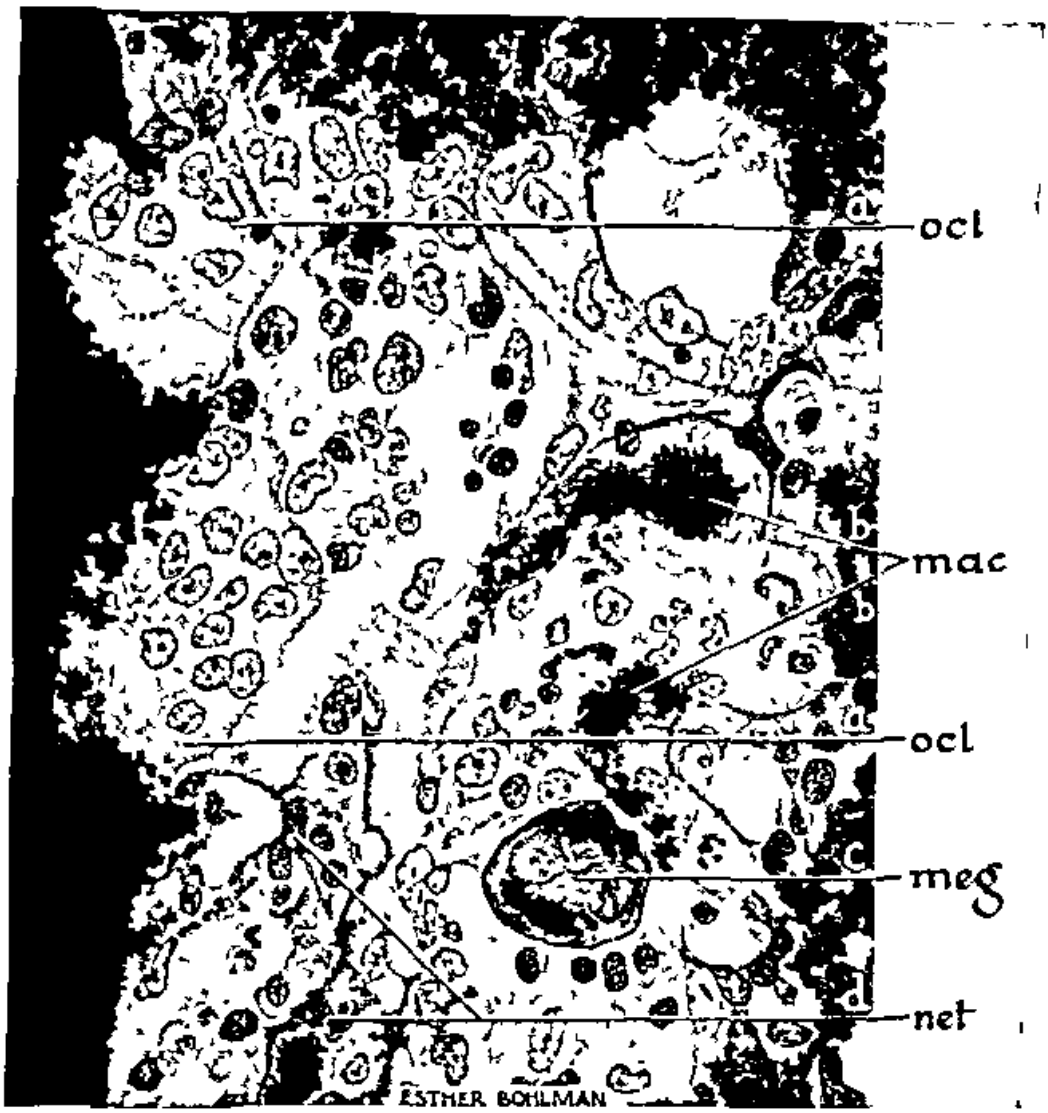

FIG 18 —Mobilization of bone salt under the influence of toxic doses of parathyroid extract High power view from undecalcified section of a rib of a puppy 12 hours after injection of 200 U.S P. units of parathyroid extract per kilogram of body weight. (*a*) Osteoclasts, free from aggregated bone salt, (*b*) macrophages, packed with bone salt, (*c*) megakaryocyte, containing bone salt, (*d*) basophil network. Formaldehyde fixation, silver nitrate–hematoxylin-eosin; camera lucida; ×681 (From original drawing of Fig 2*B*, McLean and Bloom, Arch. Path., 32:310. Reproduced by courtesy of the publishers.)

CONTROL OF OSTEOCLASTIC RESORPTION

If the view be accepted that osteoclasts resorb bone, there remains the very important subject of the control of this process, which certainly does not occur spontaneously or at random. Resorption of bone, accompanied by the presence of osteoclasts, begins with the very earliest stages of ossification in the embryo and continues throughout life, although there are marked differences in the location and rapidity of resorption, according to the physiologic and structural needs of the moment. The influences which evoke and control osteoclastic activity must be looked for, and these may be found either generally, throughout the organism, or locally, in circumscribed areas.

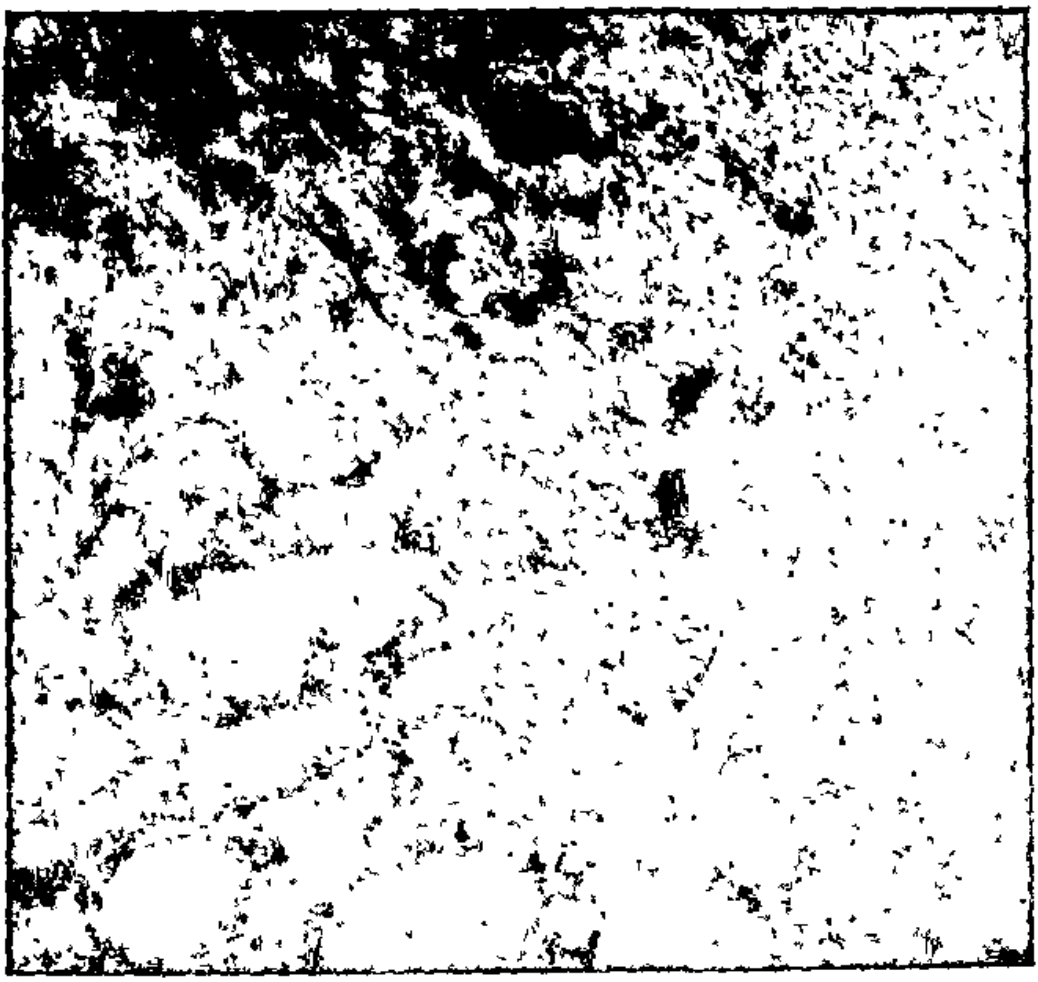

FIG. 19.—High-resolution electron micrograph showing mineral crystals from resorbing bone within the cytoplasmic vacuoles of an osteoclast. ×66,600. (From original of Fig. 18, Scott and Pease, Anat. Rec., 126:495. Reproduced by courtesy of the publishers.)

cal bone. The net result is that the cartilage disk remains at approximately constant thickness, while the diaphysis increases in length. The growth of the bone is dependent upon proliferation of the cartilage; but unless the mechanism, which we have referred to as the *growth apparatus*, functions as a whole, physiologic growth *cannot occur.*

Hypophysectomy of the immature rat leads to prompt cessation of growth, both in weight and in length. The epiphyseal disks of the long bones soon show evidence of inactivity. Within two weeks the thickness of the cartilage disk is far below the normal for the age of the animal; the cartilage cells of the disk decrease in size; the osteoblasts become spindle-shaped; and the primary spongiosa disappears. Eventually the epiphyseal cartilage disk becomes sealed from the marrow by a lamina of bone. As a result of the premature arrest of growth, the disk resembles that of the adult rat.

If growth hormone is administered to such an animal while the growth apparatus is quiescent, increase in weight and in skeletal size occurs promptly. Growth in the epiphyseal cartilage and in the adjacent spongiosa is resumed, and there is removal of the sealing lamina of bone, with re-establishment of invasion of the cartilage. The histologic appearance of the entire growth mechanism is again that of the growing animal. Similarly, if growth hormone is administered to a rat at the age of approximately five months, at a time when the growth curve has reached a plateau, reactivation of the growth apparatus, which has become quiescent, is demonstrable. There is a large volume of literature upon the effects of growth hormone on metabolic activity, both in man and in other animals. Most of this is of uncertain applicability to the subject of the growth in length of the long bones, to which our attention is restricted. For our present purposes, we are concerned with the growth apparatus as a primary target for the action of the hormone.

The results of the administration of growth hormone, derived from bovine or porcine sources, to man have been disappointing; large doses administered to patients with panpituitary dwarfism have had little effect. More recently, preparations of the hormone from primate sources, including human pituitary glands, have

CHAPTER IX

Regulatory Processes and Bone

A. Bone Matrix

Consideration of the regulatory processes affecting bone, or in which bone plays an important part, requires attention to the interplay of the physiologic factors in the growth, mineralization, turnover, and maintenance of the integrity of skeletal tissues. And since bone is so closely linked with the homeostatic control of the composition of the internal environment, more especially of its content of calcium and phosphate but including sodium and magnesium as well, it is not possible to consider the factors influencing the mineral of bone without attention to the bone-blood relationships; these are influenced by both vitamins and hormones.

GROWTH HORMONE

Of the hormones known to influence growth, including androgens, estrogens, adrenocortical hormones, thyroid, and insulin, only the growth hormone of the anterior lobe of the pituitary gland exhibits a specific effect upon the growth apparatus by means of which growth in length of the long bones continues up to closure of the epiphyses.

The anterior lobe of the pituitary gland secretes, among other hormones, a growth or somatotropic hormone that exerts an influence on skeletal growth. Until recently, the hormone has been prepared mainly from bovine sources, and the test object has usually been the rat—intact or hypophysectomized.

In the long bones, prior to closure of the epiphyses, growth in length occurs by a continuous invasion of the proliferating cartilage by capillaries and osteogenetic cells and its replacement by diaphys-

eal bone. The net result is that the cartilage disk remains at approximately constant thickness, while the diaphysis increases in length. The growth of the bone is dependent upon proliferation of the cartilage; but unless the mechanism, which we have referred to as the *growth apparatus*, functions as a whole, physiologic growth cannot occur.

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The results of the administration of growth hormone, derived from bovine or porcine sources, to man have been disappointing; large doses administered to patients with panpituitary dwarfism have had little effect. More recently, preparations of the hormone from primate sources, including human pituitary glands, have

demonstrated the importance of species specificity. Purified human growth hormone, administered to patients in minute amounts, produces growth in length of the bones and retention of nitrogen. In an early report there is one instance in which the hormone from human material, administered to a pituitary dwarf over a period of eleven months, led to a growth rate of 2.6 inches per year.

Henneman and his co-workers reported, in 1960, that they gave intramuscular injections of 0.2–10 mg. daily of human growth hormone (HGH) to ten patients and found that it produced retention of all cellular elements measured, increased calcium absorption, and probably mobilized fat. At these dose levels the chemical response waned after three to four weeks of continuous treatment; responsiveness was restored by a rest period of twelve days. Of the ten patients treated, five had open epiphyses; of these, linear growth was stimulated in three and probably in the other two. The most consistent and sensitive indices of response to HGH were decreases in urinary nitrogen, sodium, and potassium.

THYROID HORMONE

Cretinism, a form of dwarfism and mental deficiency resulting from thyroid insufficiency, has been observed for centuries. It did not become a well-established entity, apart from other forms of dwarfism, until the function of the thyroid gland was discovered; it then became known that lack of the internal secretion of this gland causes a general retardation of skeletal development and growth.

Removal of the thyroid gland, in young rats, leads to a marked slowing of growth. The skeleton resembles those of hypophysectomized rats, in its small size, but there are important differences. The epiphyseal cartilages are not sealed by bony plates; some erosion continues; and the primary spongiosa retains its characteristic appearance. Normal growth is resumed when thyroid hormone is administered. Growth in thyroidectomized rats can also be elicited by injection of growth hormone, although they are relatively insensitive to this treatment. Thyroxin injected into normal rats causes no increase in body length, but it has a synergistic effect when administered together with growth hormone. All these obser-

vations point to a non-specific effect upon the growth of bone; they may be interpreted as reflecting indirect effects of the action of the thyroid secretion on metabolism in general.

Overdoses of thyroxin in experimental animals may lead to premature closure of the epiphyses and consequent cessation of growth. The same effects may follow administration of thyroid to children in an effort to promote growth. A temporary increase in the rate of growth may occur, but the final result may be undesirable. Retardation of growth of the long bones has also been reported in clinical hyperthyroidism.

The thyroid hormone with the highest biologic activity, amounting to from five to ten times that of thyroxin is 3,5,3′-triiodothyronine; it represents 5–7 per cent of the total iodine in the gland. The mixture of four iodothyronines, of which thyroxin is the most abundant, may be considered as the physiologic hormone, since all are present and each has biologic activity.

ANDROGENS AND GONADOTROPINS

The chief source of gonad-stimulating hormones is from the placenta; these hormones appear in large amounts in the urine during pregnancy; because of their origin, they are called *chorionic gonadotropins*. Their effects are similar to, but not identical with, those of hormones elaborated by the anterior lobe of the pituitary gland. When they are administered to male animals, including man, they have certain effects upon the growth of bone, resembling those of the male hormones or androgens. Since the effects of the gonadotropins are the result of increased production of androgens, the two groups of substances will be considered together.

The long bones of rats castrated at the age of twenty-one days and killed when one year of age are significantly shorter than those of control rats. Large doses of androgens depress growth or have no effect. In the human male the influence of the gonadotropic hormones of pituitary origin is correlated with the growth that occurs normally at the age of puberty. In the female, gonadotropin is not often used to induce growth; testosterone may be used instead.

The chorionic gonadotropins are considered by some to be the

most reliable growth-promoting substances available. They have an accelerating effect upon growth during childhood, as well as at puberty, but give rise to premature development of secondary sex characteristics. Induction of sexual maturity with chorionic gonadotropins may be useful in preparation for the administration of growth hormone. Reports that they lead to early closure of the epiphyses are now discounted.

INSULIN

There is interest in the role of insulin in promoting growth, and an insulin-like action of growth hormone has also been described. By using a slow-acting insulin in gradually increasing doses, an average increase in weight of 36 gm. in fifteen days has been attained in completely hypophysectomized rats. Control rats ate less of the same diet and did not grow. These findings are believed to demonstrate that insulin may stimulate growth, and they strengthen the possibility that decreased liberation of insulin may be one of the factors limiting growth in hypophysectomized animals.

On the other hand, an effect on glucose uptake of the diaphragm from a normal rat, similar to that observed with insulin, has been demonstrated under the influence of growth hormone. The effect is absent when the rat is made diabetic, but it returns when diabetic rats are injected with insulin. It is postulated that the effect is due to liberation, by the growth hormone, of insulin held in an inactive state in blood or tissue. These observations, while only suggestive, point to the possibility that insulin may be one of the factors regulating growth.

DWARFISM

There are probably few cases of dwarfism, apart from cretinism, in which the retardation or cessation of growth is attributable to the absence of any one hormone. Interest has been centered largely on dwarfism ascribed to insufficiency of the anterior lobe of the pituitary gland. The syndrome of hypopituitary dwarfism, however, frequently includes genital retardation or infantilism and may include cryptorchidism; this syndrome is known as *panpituitary dwarfism.*

Stunting of growth resulting from hypothyroidism is easily recognized, and the treatment is specific. The differentiation between hypopituitary dwarfism, *primordial dwarfism*, and constitutional delay of growth and development is difficult in childhood; the diagnosis is often established only toward the end of the period in which growth is possible. The hypopituitary dwarf has marked delay in epiphyseal ossification and fusion but differs from the hypothyroid dwarf in that the skeletal proportions assume the mature type and that epiphyseal dysgenesis does not occur. The primordial or *genetic dwarf*, represented by the *Pygmy*, has relatively normal epiphyseal development and exhibits no gonadotropic or other hormonal deficiency. The dwarfism that occurs as the result of *ovarian agenesis* resembles the primordial type.

As indicated above, growth hormone from human pituitary glands has produced linear growth and retention of nitrogen, calcium, sodium, and potassium in human subjects. Species specificity has been demonstrated, and the efficacy of the hormone from primate sources no longer remains in doubt. Further progress in this respect will depend upon developing adequate sources of material that can be used in patients with pituitary dwarfism.

Chorionic gonadotropin and preparations of gonadotropin from pituitary sources are in use for treatment of sexually immature dwarfs; thyroxin and androgens are also of practical value. Gonadotropins, when applicable, are preferable to sex hormones for young patients because they stimulate rather than depress gonadal function; as a rule, testosterone is preferred in females. Synthetic hormones, such as diethylstilbestrol, are as effective as natural female sex hormones; when administered in cycles they are helpful in dwarfism associated with ovarian agenesis.

Because of the undesirability of inducing secondary sex characteristics prematurely, gonad-stimulating therapy should not begin until the patient is about fourteen years of age. The consensus now is that premature epiphyseal closure, following administration of gonadotropins, need not be feared; contrary results are reported after testosterone.

HORMONAL REGULATION OF INTEGRITY OF ORGANIC MATRIX OF BONE

Since physiologic turnover affects both the mineral and organic constitutents of bone, it follows that there must be continuous destruction and rebuilding of the organic matrix. It appears that collagen itself is slightly, if at all, subject to metabolic turnover; it requires replacement only when destroyed by resorption. Once resorption has occurred, rebuilding follows; this is best seen in the haversian remodeling of compact bone. There is an increasing body of evidence that this rebuilding is under hormonal control and, indeed, that there exists an antianabolic as well as an anabolic factor. A current view is that the sex hormones—androgens and estrogens—provide the anabolic factors and that the adrenocortical hormones—cortisone and hydrocortisone—are antianabolic in their effects. According to this view there is a balance between the production of the sex hormones and the adrenocortical hormones which, under normal circumstances, results in a balance between matrix destruction and matrix rebuilding

The integrity of the organic matrix, as well as its normal growth and development, depends also upon an adequate intake of vitamins A and C. For this reason the influences exerted by these vitamins upon bone are considered together with those of the hormones.

ESTROGENS AND BONE

Steroid hormones, both natural and synthetic, that are capable of producing estrus, also lead in certain experimental animals to specific effects upon bone. More than forty compounds with estrogenic activities are known, of which estradiol and estrone have been isolated from the ovaries and are the prototypes for the group.

Present knowledge concerning the relationship of estrogens to postfetal osteogenesis is obscure in all animals except birds. In 1934, Kyes and Potter observed that female pigeons, during the preovulatory phase of the egg-laying cycle, produce a secondary system of spongy bone arising from the endosteum and growing into the marrow cavity. These intramedullary deposits of new bone, a

secondary sex characteristic, develop under the influence of estrogens, and serve the purpose of storing calcium to be used later for calcification of the egg shell. Intramedullary bone develops naturally in the females of all avian species studied; it may also be produced in either males or females, by administration of estrogens. In mice a similar reaction can be produced by estrogen injections, but it does not occur naturally during reproduction. Still another reaction to estrogen, that of inhibition of resorption of the spongy bone of the metaphysis, can be seen in the growing rat.

The role of estrogen in the regulation of calcium and bone metabolism is more highly developed and easier to study in the domestic chicken than in any other animal. In the laying hen estrogen controls, simultaneously, the formation of protein and its transport together with calcium for the developing egg yolk, as well as the formation and calcification of intramedullary bone, for later use in the calcification of the egg shell. During the initial fourteen-hour phase of the egg-laying cycle, the plasma calcium may rise to three times the normal level. The calcium added to the serum is bound to a phosphoprotein (X_1) as a non-ionized, non-ultrafilterable complex. This is produced by the liver, in association with a lipoglycophospholipid lipoprotein (X_2). The two proteins are very large molecules in transport in the plasma and serve as precursors to the granules of the egg yolk. These proteins do not exist in the plasma without a large complement of calcium, and they are dependent upon calcium for transportation from the liver to the yolk sac in the form of osmotically inactive units. The hypercalcemia of X_1–X_2 proteins is also found during egg production or following estrogen treatment in the plasma of fish, frogs, turtles, and viviparous snakes (Fig. 20).

It is the diffusible and not the protein-bound fraction of the plasma calcium that is concerned with calcification of the egg shell. During the final ten hours of the egg-laying cycle, especially at night when the bird is not feeding, the egg rests in the uterus for deposition of the calcified egg shell. During this time, by some stimulus that is not known, but not by action of the parathyroid hormone, the calcium is mobilized from the intramedullary bone

which undergoes extensive resorption. The calcium is transferred from the skeleton to the egg shell and is redeposited as calcium carbonate; the phosphate is excreted in the urine.

It is easy to follow the transformations of the cells in pigeons during the rapid and extensive changes in the bone marrow under

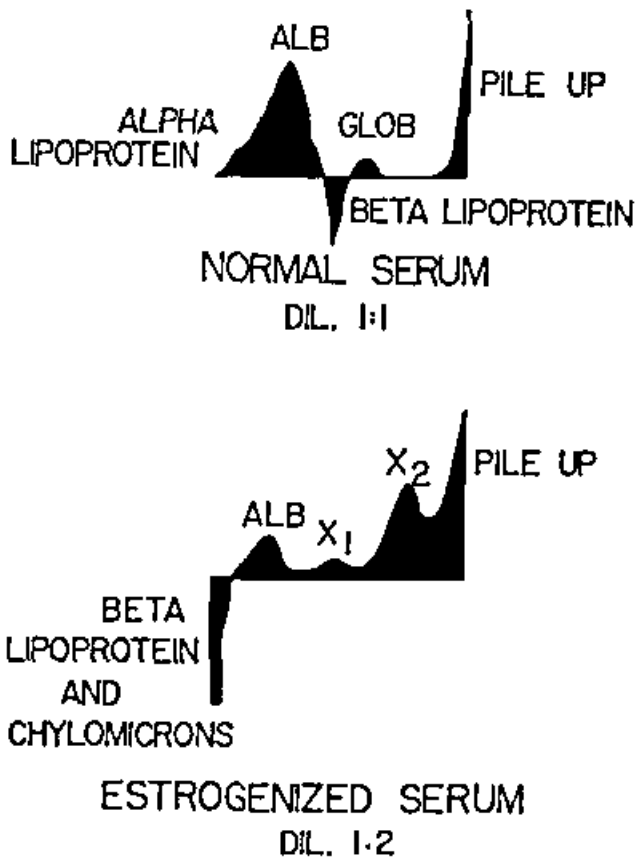

FIG 20.—Composite schlieren patterns of the serum (*above*) of a normal rooster, and (*below*) of a heavily estrogenized rooster. X_1 and X_2 are new components secreted by the liver into the plasma in the form of complexes with calcium. (From original of Fig. 1, Urist, Schjeide, and McLean, Endocrinology, 63:573. Reproduced by courtesy of the publisher.)

the influence of estrogen. During the preovulatory bone-forming period, reticular cells become osteoblasts and then, in turn, become osteocytes. In the bone-destroying phase after ovulation of the first egg, osteoblasts and liberated osteocytes become osteoclasts; the osteoclasts may turn into osteoblasts during the second period of bone formation. In the post-ovulation period, osteoblasts and osteoclasts again become reticular cells.

When estrogens are administered to mice, a similar, although not identical, reaction begins in the spongy bone of the metaphysis and grows into the marrow cavity by direct extension. Newborn mice do not respond, but after ten days of growth and until senility the skeleton is always capable of producing large amounts of endosteal bone. Within the limits of tolerance of the animal to the hormone, the yield of new bone is proportional to the dose and the period of administration; it may eventually fill the entire marrow cavity (Fig. 21). There is no inhibitory or synergistic action by progesterone, deoxycorticosterone, or testosterone or by anterior pituitary growth hormone, gonadotropic hormone, or adrenocorticotropic hormone. Experiments with C^{14}-labeled estrone indicate that there is a selective deposition of the hormone in the endosteum; its further fate is not known.

The formation of endosteal bone in the mouse under the influence of estrogens has not been shown to reflect any physiologic function. Bone formation is not followed by rapid resorption as it is in the bird; when administration of the hormone is discontinued, there is gradual removal of the excess bone. During pregnancy, when large amounts of estrogens are being excreted in the urine, neither the fetal nor the maternal skeleton exhibits medullary bone formation. Nor has it been possible to induce new-bone formation in either mature or immature mice by stimulating the animal's own ovaries by large doses of gonadotropic hormones. Moreover, estrogens implanted in the bones, in the form of pellets, produce only the usual systemic effects, with no special local reaction. During the period of new-bone formation the serum calcium, inorganic phosphate, and alkaline phosphatase levels remain within normal limits.

Administration of estrogens to young, growing rats leads to superficially similar, but essentially different, effects from those seen in birds and in mice. No formation of new bone is observed, but the resorption of metaphyseal bone is inhibited, with the result that for a distance of several millimeters in the marrow cavity there is an elongated core of unresorbed spongy bone (Fig. 22). The effects of estrogens on the bones of the rat are limited to this specific action upon one part of the growth apparatus, concerned only with

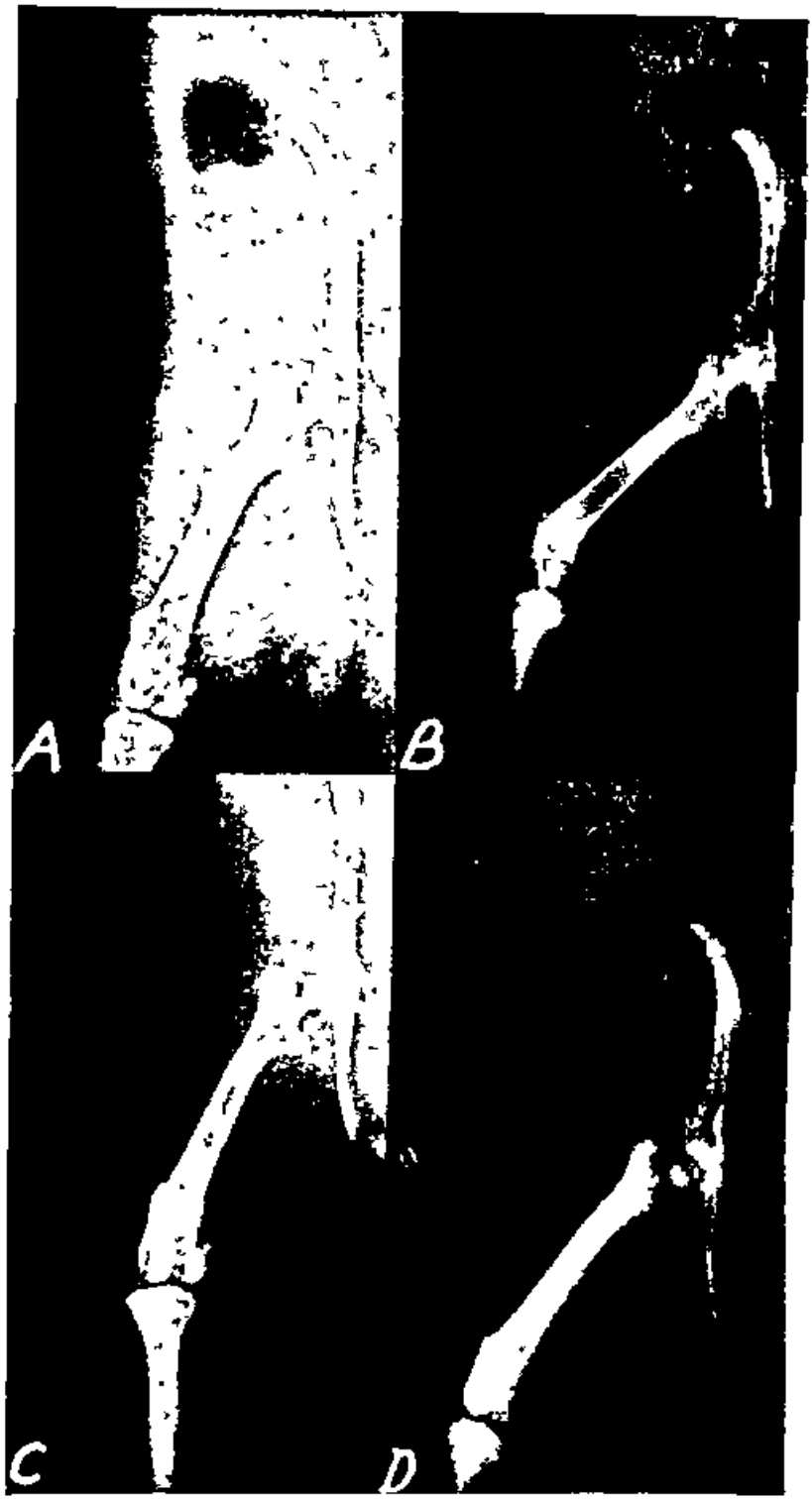

FIG. 21.—Roentgenograms of femurs of mice to illustrate formation of new bone within marrow cavity under influence of substances with estrogenic activity. Littermate CF_1 mice treated with large doses of diethylstilbestrol in corn oil. *A*, control, injected with 0.4 cc. of corn oil, *B*, 6 0 mg. in 5 weeks; *C*, 14 0 mg. in 15 weeks; *D*, 16 0 mg. in 17 weeks. The volume and distribution of the new bone can be determined as accurately by roentgenograms as by histologic sections. (From originals of Fig. 3, Urist, Budy, and McLean, J. Bone & Joint Surg., 32A: 146. Reproduced by courtesy of the publishers.)

endochondral growth. Consequently, as is to be expected, no demonstrable effects are found in adult rats, and intramembranous ossification is not affected at any time.

Lindquist *et al.* have shown, by a combination of autoradiography and the *kinetic analysis* of Bauer, Carlsson, and Lindquist,

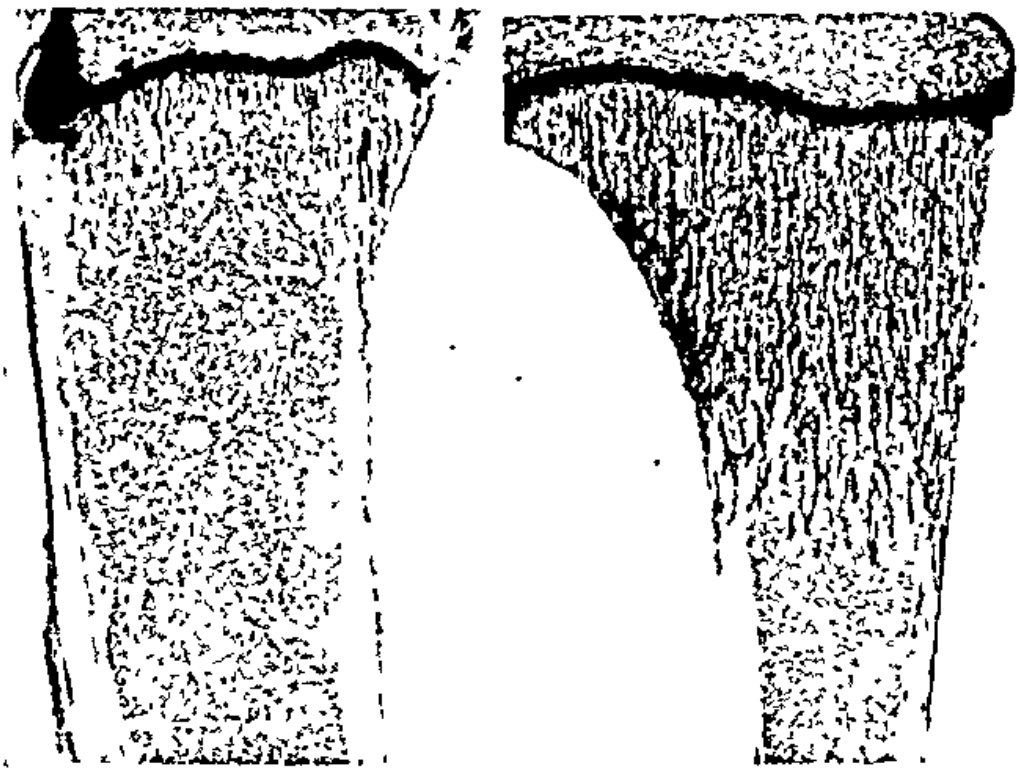

FIG. 22.—Sections to illustrate inhibition of resorption of spongiosa, with consequent increase in length, following administration of estrogens to immature rats. Photomicrographs of the proximal ends of tibias. *Left*, control, *right*, increase in the length of the spongiosa up to 6–8 mm. resulting from administration of 8.0 mg. of 17-β estradiol benzoate over a period of 5 weeks. Hematoxylin-eosin-azure II. ×9.8. (*From original of* Fig. 2, Urist, Budy, and McLean, Proc. Soc. Exper. Biol. & Med., 68:325; and Figs. 1 and 4, Budy, Urist, and McLean, Am. J. Path., 28:1157 and 1163. Reproduced by courtesy of the publishers.)

that the inhibition of resorption by estrogen in the young rat affects only the cores of secondary spongiosa, as observed in the long bones; the resorption incident to growth in length and the accompanying remodeling is not influenced by the hormone.

Guinea pigs, hamsters, rabbits, kittens, and puppies have failed to show any specific skeletal response to administration of estro-

gens. Administration of these hormones to man, particularly in the presence of *osteoporosis*, has led to equivocal results; there seems to be no basis for extrapolating the effects on birds, mice, and rats to man or to any species other than those named.

CORTICOTROPIN (ACTH) AND ADRENAL CORTICAL STEROIDS

Many steroids, some with biologic activity, have been isolated from the adrenal cortex; others, with similar activities, have been synthesized and are widely used in medicine. Those concerned in gluconeogenesis are classified as gluco-corticoids; they have systemic regulatory physiologic effects. The naturally occurring gluco-corticoid with the most important action on the skeleton in man is *hydrocortisone* (17-hydroxycorticosterone). Elaboration of this substance by the adrenal cortex, as well as of *cortisone* (11-dehydro-17-hydroxycorticosterone), is under the control of the anterior lobe of the pituitary gland, mediated by secretion of the *adrenocorticotropic hormone* (corticotropin; ACTH). Production of gluco-corticoid hormones by the adrenal cortex continues after hypophysectomy, but at a reduced rate.

Insofar as the adrenal corticosteroids affect the growth of bone, the effects appear to be non-specific and secondary to the systemic regulatory functions of the hormones. Overdosage, either of ACTH or of the gluco-corticoids, leads to loss of nitrogen in the urine and feces, attributed to an interference with the metabolism of proteins. In young growing animals there is also an adverse effect upon the growth apparatus, resulting in a slowing of growth. Similarly, retardation of healing of experimental fractures in animals, as a result of administration of gluco-corticoids, is attributed to depression of osteoblastic activity.

The adrenal gluco-corticoids assume major importance in relation to the skeleton in *Cushing's syndrome* (*hypercortisonism*), in which there is hypersecretion of the corticosteroids. This was originally attributed to a basophil adenoma of the pituitary gland, but overactivity of the adrenal cortex may result from other causes or may be associated with a primary hyperplasia or tumor of the

cortex itself. The condition has a characteristic constellation of symptoms; the effect upon the skeleton is a marked loss in the mass of bone (*osteoporosis; osteopenia*); often accompanied by fractures. Similar effects are reported following long-continued administration of exogenous corticosteroids, either natural or synthetic, although there is not a one-to-one correlation between dosage and skeletal effects. There are continuing efforts to modify the steroid molecule in such a way as to enhance the desirable physiologic or therapeutic effects, while reducing the undesirable side effects, such as osteoporosis; in no instance has it been demonstrated that the risk of osteoporosis can be lessened by use of newer compounds. The relationship of the corticosteroids to osteoporosis is treated at length in a subsequent chapter.

The adrenal cortex also secretes substances with mineralo-corticoid activity, of which the most important is *aldosterone;* its role in mineral metabolism is considered in connection with homeostasis and control of the electrolyte pattern of the internal environment.

VITAMIN A

The term vitamin A has been applied to at least five substances that produce a characteristic response in the animal body. There are vitamin A itself, found exclusively in the animal organism, and the provitamins A or carotenoid pigments which are converted to vitamin A in the intestinal tract of animals. The most important naturally occurring provitamins A are α-, β-, and γ-carotenes and cryptoxanthin, of which β-carotene is most widely distributed in nature; it is found in association with chlorophyll in all green plants.

Utilization of vitamin A involves cellular metabolism, particularly in epithelial cells; its formation from precursors; storage and mobilization; absorption and transport; and specific function in vision. The vitamin plays an important role in the metabolism and growth of bone tissue. It is essential for the activities of epiphyseal cartilage cells, for without it they cannot carry out the sequence of growth, maturation, and degeneration of the growth apparatus; this failure results in suppression of endochondral growth of bone.

Remodeling sequences also cease to operate; appositional growth of bone of periosteal origin continues. These effects lead to abnormalities in the shape of the bones; the failure of certain bony foramina to enlarge brings about pressure on various nerves, resulting in their degeneration.

Excessive amounts of vitamin A lead to fragility and subsequent fractures in the long bones. These effects result from excessive osteoclastic resorption, particularly in regions of active remodeling. Fragments of crystalline vitamin A acetate have been attached to small pieces of parietal bone and implanted into the cerebral hemispheres of littermate rats. Resorption of the grafts, accompanied by numerous osteoclasts and often leading to perforation of the bone, was apparent within fourteen days. This is interpreted to indicate that vitamin A, like parathyroid hormone, has a local action on the osteoclastic resorption of bone; this is not accompanied by any effect on the serum calcium level.

Information about tissue changes resulting from vitamin A deficiency is based on observations on infants and young children, where previous storage of the vitamin is limited; it is not usually a simple deficiency state. Avitaminosis A rarely occurs in adults, and then only in association with a very prolonged intake of inadequate and unbalanced diets deficient in many dietary essentials. Since the effects of vitamin A deficiency may be prevented by very small amounts of the vitamin in the diet, and since hypervitaminosis A results only from ingestion of very large amounts, neither disorder is likely to occur except under experimental or unusual conditions.

VITAMIN C

Vitamin C, or ascorbic acid, is widely distributed in the plant and animal kingdoms; it is present in abundance in many fresh vegetables and fruits but is entirely lacking in the common cereals and grains. All pure vitamin C used in pharmaceutical products is prepared synthetically. It is a dietary essential for man, other primates, and the guinea pig but can be synthesized by other species. In animal tissue the highest concentration of the vitamin is found in the adrenal cortex, eye lens, and liver tissues.

The metabolic consequences of experimental vitamin C deficiency in susceptible animals are restricted to tissues of mesenchymal origin and are characterized by failure of formation and maintenance of intercellular materials. All intercellular substances of the supporting tissues—bone, cartilage, fibrous connective tissue, and dentin—have in common a matrix largely made up of collagen, and it is this material that in *scurvy* either is not produced or is produced in defective form. As to bone, deposition of the matrix ceases and osteoblasts assume the form of reticular cells. The mechanism of calcification itself is not affected, but abnormal connective tissue formed in bone during vitamin C deficiency is not calcifiable. The effects of the deficiency are reversible and are rapidly corrected when ascorbic acid is supplied.

There is no evidence that the vitamin has any deleterious effects when supplied in excess, and there is no rationale for its use in any conditions other than its deficiency.

also exercises control over the excretion of phosphate by the kidneys and aids in influencing the absorption of calcium in the gastrointestinal tract. Vitamin D, on the other hand, is the major influence upon the absorption of calcium; by this means it controls the mineralization of bone by assuring an adequate supply of the necessary materials. Vitamin D also exerts an effect upon mobilization of mineral from bone, complementing the action of the parathyroid hormone in this respect. An oversimplified version of current views would be to say that vitamin D influences mobilization, in proportion to its formation and intake, and that the parathyroid glands are called upon to balance the interplay between dietary intake, absorption, urinary and fecal excretion of calcium, and the regulatory effect of vitamin D. In support of the view of a synergistic action of vitamin D and the parathyroid hormone it may be mentioned that both promote the formation of citrate in bone, although by different mechanisms, and that both thus contribute to whatever effect bone citrate may have upon the transfer of calcium from bone to blood.

The enlargement of the parathyroid glands observed in rats on diets deficient in both calcium and vitamin D, and believed to result from the deficiency in calcium, has been attributed by Crawford *et al.* to the deficiency in vitamin D, since hypertrophy of the parathyroids did not occur when the rats were fed diets very low in calcium, but with an adequate supplement of vitamin D. Bloom *et al.*, however, have reported that hypertrophy of the parathyroid glands occurred when laying hens were placed on a diet low in calcium but adequate in all other components, including vitamin D. While the state of the parathyroids, in the two species, cannot be correlated with the vitamin D intake, there is good correlation between the plasma calcium levels and the size of the parathyroids. In the rats the serum calcium was maintained at near normal levels by the action of vitamin D, at the expense of the skeleton; in the hens, however, the serum calcium was markedly depressed, in the presence as well as in the absence of vitamin D. The conclusion seems warranted that hypertrophy of the parathyroids was the result of a low plasma calcium level, rather than a direct result of

CHAPTER X

Regulatory Processes and Bone B. Vitamin D–Parathyroid Complex

Higher organisms are possessed of systems which comprise mineralization and resorption of bone in addition to exchange of the ions of the bone mineral with the fluids of the body. Closely allied with these physiologic activities is the homeostatic control of the calcium ion concentration in the blood plasma and in the other extracellular fluids. In the functioning of these systems, the organism controls absorption of calcium and phosphate and their excretion, making use of the gastrointestinal system and the kidneys, both for conservation of needed elements and elimination of any excess. Operation of such complex and interrelated functions requires integration of regulatory mechanisms, under central and humoral control. Of the controls, the most important, insofar as the metabolism of calcium and phosphate is concerned, are vitamin D, both endogenous and exogenous, and the parathyroid hormone. So closely interrelated are the functional activities of the parathyroid hormone and of vitamin D that it will serve our purposes to regard them both as component parts of an integrated system, which exercises control over the mechanisms required for proper functioning of the organism as a whole.

It was suggested very early that the vitamin and hormone might act by influencing each other; this, however, has never been demonstrated, and it is now assumed that each makes its own contributions to the regulatory processes. To state these briefly, the parathyroid glands monitor the calcium ion concentration in the plasma and apply the necessary corrections, mainly by influencing the mobilization of calcium from the skeleton; the parathyroid hormone

also exercises control over the excretion of phosphate by the kidneys and aids in influencing the absorption of calcium in the gastrointestinal tract. Vitamin D, on the other hand, is the major influence upon the absorption of calcium; by this means it controls the mineralization of bone by assuring an adequate supply of the necessary materials. Vitamin D also exerts an effect upon mobilization of mineral from bone, complementing the action of the parathyroid hormone in this respect. An oversimplified version of current views would be to say that vitamin D influences mobilization, in proportion to its formation and intake, and that the parathyroid glands are called upon to balance the interplay between dietary intake, absorption, urinary and fecal excretion of calcium, and the regulatory effect of vitamin D. In support of the view of a synergistic action of vitamin D and the parathyroid hormone it may be mentioned that both promote the formation of citrate in bone, although by different mechanisms, and that both thus contribute to whatever effect bone citrate may have upon the transfer of calcium from bone to blood.

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a deficiency in vitamin D. This seems to negate earlier conclusions that vitamin D acts through its influence upon parathyroid activity.

VITAMIN D

Numerous contributions on the role of vitamin D in calcium metabolism, and on the mode of its action, are now appearing, including studies at the cellular level. Vitamin D, whether endogenous or exogenous, may be regarded as essential to the total system which includes calcium metabolism and mineralization of bone; its effects are made manifest in a variety of ways.

The term vitamin D was originally applied to the factor in cod liver oil responsible for its antirachitic potency. It is now used to refer to two or more fat-soluble steroids with antirachitic activity and also the ability to elevate the serum calcium level, by influencing the mobilization of mineral from bone. Two forms of vitamin D are well characterized chemically. Vitamin D_2, or calciferol (ergocalciferol) is obtained as one of the products of activation of ergosterol. Vitamin D_3 (cholecalciferol) is the naturally occurring antirachitic factor in cod liver oil; it can also be prepared by activation of 7-dehydrocholesterol. These two forms of vitamin D possess equal antirachitic potency in man. Dihydrotachysterol is closely related to calciferol; it has negligible antirachitic potency but has calcemic activity. The current trend is to regard it as a form of vitamin D. Since there are now several sterols possessing antirachitic and calcemic properties, in varying proportions, chemical identification and characterization should be the basis for nomenclature.

The antirachitic activity of vitamin D, in prophylactic doses, is effected mainly by its influence upon intestinal absorption of calcium. In very large doses, 50,000 I.U. or more daily, vitamin D exhibits a marked calcemic effect, resulting in an increased concentration of calcium in the blood plasma—an effect similar to that produced by parathyroid extract. This subtoxic action of the vitamin and of other chemically related sterols is made use of to raise the level of serum calcium in hypoparathyroidism. In even larger doses the effects are manifestly toxic and result in *hyperi-*

taminosis D. This is associated with resorption of bone, comparable to that induced by large doses of parathyroid extract. In the young rat there follows a marked increase in the trabeculae of the spongy bone of the metaphysis. This new bone is calcified poorly or not at all, in spite of hypercalcemia and a consequent elevation of the Ca × P product; this is the result of production of an uncalcifiable matrix and has been designated as *hypervitaminosis D rickets.*

Although the earlier idea of a direct effect of vitamin D on bone, by which calcification is promoted, has been largely supplanted by the view that the direct effect leads to mobilization of calcium, rather than to its deposition, there are still adherents to the earlier view; Migicovsky has continued exploration of this possibility, with equivocal results.

PARATHYROID HORMONE

The parathyroid glands supply an internal secretion, the parathyroid hormone, intimately concerned with the regulation of the calcium ion concentration in the blood. The primary target of the hormone, in this respect, is the skeleton; regulation of the plasma calcium level is also indirectly aided by an effect upon tubular reabsorption of phosphate by the kidneys. Enhancement of active transfer of calcium across the gut wall by parathyroid hormone has been demonstrated *in vitro;* there is also an influence of the hormone upon the secretion of calcium by the lactating mammary glands of rats. A further extension of parathyroid activity to connective tissue in general, throughout the organism, with its chief effect upon the mucopolysaccharides of the ground substance, has been postulated; this effect, if any, is poorly understood. While a primary effect of the hormone is upon mobilization of calcium from the bone mineral, there is no evidence that it influences deposition of the mineral.

Ablation of the parathyroid glands leads to *hypoparathyroidism,* with a lowering of the calcium ion concentration in the blood, frequently accompanied by tetany. An excess of parathyroid hormone, either endogenous or exogenous, leads to an increase in the calcium ion concentration in the blood and may produce profound changes in the skeleton.

NATURE OF PARATHYROID HORMONE

Solution of many of the problems concerning the physiologic activity of the parathyroid hormone is being greatly aided by the use of a pure preparation of the active principle from the glands. This has enabled chemical characterization of the hormone, and use of the pure preparation avoids the undesirable features of the crude extracts previously available.

Rasmussen and Craig have isolated and characterized a single protein with a molecular weight of approximately 9500, which is homogeneous by countercurrent distribution, paper and column chromatography, and ultracentrifugation; contains no cystine; and possesses a single end-terminal amino acid, alanine. The amino acid composition of the pure bovine hormone is known. The present evidence indicates that this hormone consists of a single polypeptide chain, which can be partially hydrolyzed without complete loss of biologic activity. The pure preparation has both calcium-mobilizing and phosphaturic activity; there is now no support for the view that the parathyroid glands elaborate two hormones.

EVOCATION OF SECRETION OF PARATHYROID HORMONE

The homeostasis of calcium constitutes a special case among the cations of the blood plasma. Homeostatic control of the concentrations of other cations of the fluids in the body, and of their retention or excretion by the kidneys, is exerted by integration of neural and hormonal factors. The parathyroid glands, insofar as is known, act as the receivers of information concerning the calcium ion concentration in the plasma and themselves respond, by elaboration or suppression of secretion of the parathyroid hormone, to apply any necessary correction. Their function is thus accomplished without participation of the central nervous system, the adenohypophysis, or the adrenal cortex; the existence of a parathyrotropic hormone, elaborated by the anterior lobe of the hypophysis, is no longer supported by any evidence, and the idea of such a direct link between the pituitary and the parathyroids has disappeared from modern endocrinology. Patt and Luckhardt, in 1942, were able to demonstrate, by perfusion of the parathyroid glands with

serum depleted of calcium, that these glands respond directly to a lowered concentration of calcium in the plasma by increased activity. All current evidence supports this observation.

It is believed by some that the concentration of organic phosphate in the plasma also exerts an influence upon the secretory activity of the parathyroid glands; according to this view, an increase in phosphate concentration in the plasma may lead to increased secretion of the parathyroid hormone. Such evidence for this as exists is indirect and inconclusive; now that it has been demonstrated that the parathyroids secrete only one hormone, having both calcemic and phosphaturic activity, the case for a second stimulus, acting through the parathyroids, is weakened.

MODE OF ACTION OF PARATHYROID HORMONE ON BONE

Almost from the discovery of the active principle of the parathyroid glands and of the influence of hyperparathyroidism on the skeletal system there was a considerable difference of opinion concerning the mode of action of this hormone on bone. One view was that the effect on bone was secondary to an increased excretion of phosphate in the urine; the other was that the hormone acted specifically on bone, leading to resorption with solution of the bone salt.

These differences may now be regarded as resolved. The parathyroid hormone acts directly *both* on bone and on the kidneys; current effort in this area is directed toward elucidating the effects of the hormone on cells, and toward integration of the major effects into a single system responsible for what is recognized as the primary function of the parathyroids, i.e., homeostatic control of the blood calcium level. In view of the intimate association between the physiologic activities and functions of vitamin D and the parathyroid hormone, such an integrated system must incorporate both the vitamin and the hormone.

The conclusion that there is a direct action of the hormone on bone rests on: (1) histologic examination of the bones of animals treated with parathyroid extract; the changes in the bones, described as characteristic of the toxic action of the hormone, are

observed even when the kidneys of the animals have been previously removed; (2) observation that in dogs treated with parathyroid extract the serum calcium continues to rise after a Ca × P product in the plasma is attained sufficient to induce calcification in rachitic cartilage *in vitro;* and (3) demonstration of a direct effect of parathyroid transplants on bone in contact with them. The degree to which the histologic effects of toxic doses of the hormone reflect its action in physiologic concentrations is still in doubt. The observation of changes in bone in contact with transplants, however, supplies a link between strictly physiologic conditions and those in the toxic range.

Additional convincing evidence concerning the mode of action of the parathyroids on bone has been derived from experiments in which the hormone was used to bring about an increase in the concentration of calcium and, hence, of the Ca × P product in the serum of dogs. The serum of untreated adult dogs will not induce *in vitro* calcification in the matrix of the hypertrophic cartilage of rachitic rats; such calcification may, however, be induced when the Ca × P product is raised either by adding calcium or phosphate to the serum, or by previous treatment of the dog with parathyroid extract (Fig. 23). The conditions critical for calcification, variously defined as saturation, supersaturation, or metastability, have been used as indicators of the limits of biologic solubility. These limits are exceeded when hypercalcemia is brought about by an excess of parathyroid hormone; on recovery from the calcemic effects of the hormone the serum of the dog will no longer induce calcification of rachitic cartilage *in vitro.* This we regard as conclusive evidence in favor of a biologic mechanism for solution of the bone mineral, under influence of parathyroid extract; no salt will dissolve beyond the limits of its solubility when its solution is in contact with its solid phase.

Evidence for an action of the parathyroid hormone on the renal tubules is equally convincing; the net result is an increase in the excretion of phosphate in the urine. The possibility that this is the result of a hemodynamic effect on glomerular filtration has been excluded by observations with the pure hormone; the purified hor-

mone has been shown to be devoid of the hemodynamic effects often observed following administration of crude parathyroid extract. The effect on excretion of phosphate has been attributed to decreased tubular reabsorption; an effect on tubular secretion has not been excluded.

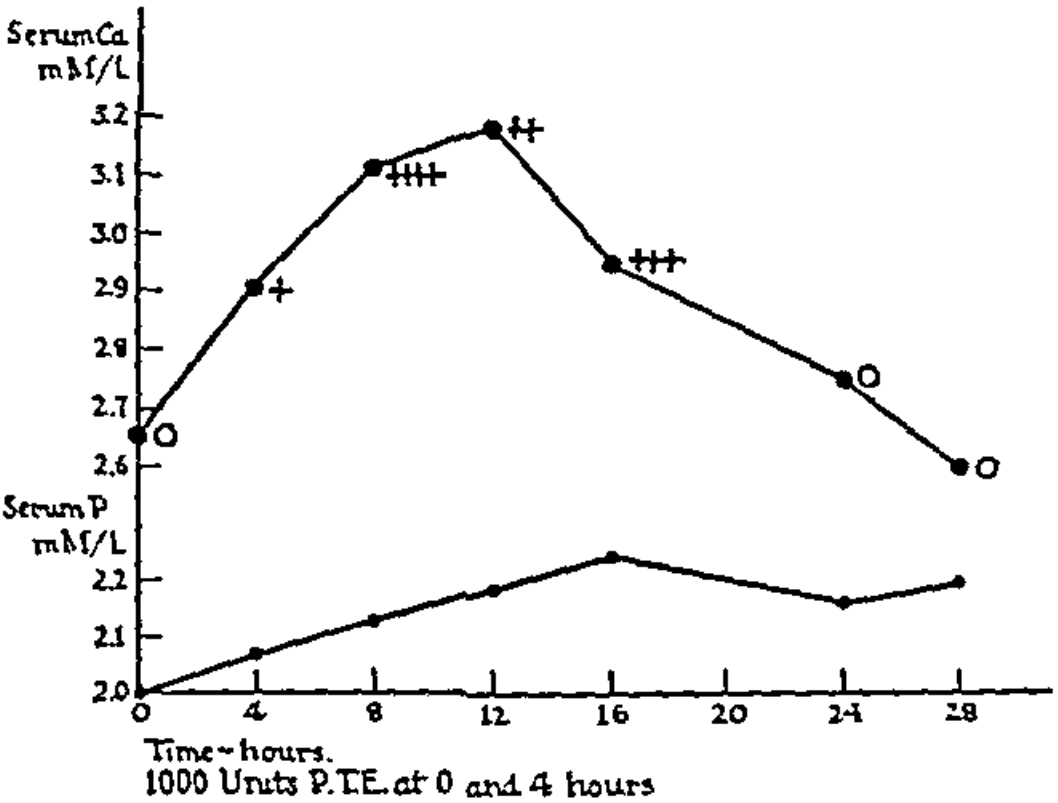


FIG 23.—Influence of concentrations of serum calcium and serum phosphate of a dog treated with parathyroid extract upon calcification of cartilage of a rachitic rat when incubated with serum. The dog was given 1,000 U.S.P. units of parathyroid extract at 0 hour, and again at 4 hours. Serum of blood withdrawn at 0, 24, and 28 hours produced no calcification of rachitic cartilage in vitro; serum of blood withdrawn at 4, 8, 12, and 16 hours produced +, ++++, ++, and +++ calcification, respectively. (From Fig. 4, McLean, Lipton, Bloom, and Barron, Tr. Conf. Metab. Aspects Convalescence, 14:31. Reproduced by courtesy of the publishers.)

NATURE OF CELLULAR ACTION OF PARATHYROID HORMONE ON BONE

There is agreement that the influence of the parathyroid hormone on bone, as well as its effects upon other tissues, such as kidney tubules, intestinal epithelium, and the mammary glands, depends on an action upon cellular constituents; there is *now no*

suggestion that the hormone *directly* influences the solubility of the bone mineral in the fluids of the body. In bone, an action could be upon any one or more of the cells of bone—osteoblasts, osteocytes, or osteoclasts.

Neuman and his collaborators have introduced a unifying concept of parathyroid hormone action, which concludes that all aspects of the action of the parathyroid hormone can be explained in terms of its ability to enhance the transport of inorganic phosphate into cells with, in bone, an associated increase in acid production by glycolysis. To apply this postulate to the influence of the parathyroid hormone upon the transfer of exchangeable calcium from bone to blood, in terms of the localization of the labile pool of calcium in the new and incompletely calcified osteons, requires only emphasis on the glycolytic cycle in these cells, and enhancement of the solubilization of bone mineral by the effects of the acid so produced. To apply it to osteoclastic resorption, which occurs at a different place, and under different conditions, in that it results in resorption of whole bone, as well as solubilization of the stable fraction of the bone mineral, requires a further explanation. This has been supplied, also by Neuman and his collaborators, who have developed a chemical view of osteoclasis based on studies with radioactive yttrium. They conclude that osteoclasts are required for the removal of bone, and that the process requires both acid, or other solubilizers of bone mineral, and proteolytic enzymes. This view of osteoclasis does not differ, in theory at least, from that advanced in this volume.

HOMEOSTATIC REGULATION OF CALCIUM ION CONCENTRATION IN BLOOD

It is firmly established that the parathyroid glands play a decisive part in the homeostatic regulation of the calcium ion concentration in the blood plasma. There is a direct correlation between the calcium levels in the body fluids and the state of activity of the parathyroid glands. At all levels, however, a rapid exchange of calcium and phosphate occurs between the blood and bones; even in the absence of the parathyroid glands a relatively constant,

although low, concentration of calcium ions is maintained in the blood. Thus while the parathyroid glands are responsible for monitoring the calcium ion concentration in the plasma, and while the state of their activity is critical for the maintenance of a normal level of these ions, other factors enter into the regulatory mechanism and must be taken into account.

ROLE OF PARATHYROID GLANDS IN HOMEOSTASIS

Once it was established, only a generation or so ago, that the parathyroid glands regulate the release of calcium from the bones into the blood, it was commonly believed that this relatively slow-acting mechanism was adequate to maintain a physiologic level of calcium ions in the plasma. Introduction of tracer methods, however, and increasing understanding of the fine structure of bone have made it clear that the parathyroid mechanism, while responsible for hour-to-hour or day-to-day adjustments, is not alone adequate for the minute-to-minute interplay between blood, interstitial fluid, and bone. Especially revealing in this respect is the demonstration that, in young animals, the turnover of the blood calcium may amount to as much as 100 per cent per minute, which is to say that the equivalent of the total amount of calcium in the blood may be replaced every minute.

As pointed out by Rasmussen, any integrated concept of the role of the parathyroid glands in homeostatic control of the calcium ion concentration must take into account not only the direct effect of the parathyroid hormone on osteoclastic resorption of bone, but also the other mechanisms and controls used by the organism to effect the fine adjustment required. Of the mechanisms under parathyroid control, Rasmussen has redirected attention to the kidneys, and to the effect upon calcium ion activity in the plasma of the changes in ion activity brought about by renal excretion of phosphate; he believes that the rapid action on the kidneys has a buffering effect upon fluctuations which would be corrected more slowly by the direct effect upon bone. The effect of the hormone on absorption of calcium by the gastrointestinal tract undoubtedly aids homeostasis. It is important also to emphasize the buffering

effect of the extracellular fluids; calcium from this source is the first to reach the blood plasma in the event of depletion; this operates also in the opposite direction, and any excess of calcium in the plasma is rapidly transferred first to the extravascular fluid.

DUAL MECHANISM

Except for the action of the hormone on bone, liberating calcium into the blood by control of osteoclastic resorption, the most important factor in maintaining the stability of the calcium ion concentration in the plasma is the pool of exchangeable calcium of the bone. Transfers between the blood and bone, buffered by the extravascular fluids, are effected rapidly in both directions; opinion differs concerning the influence of the parathyroids on this transfer in the direction of bone to blood.

In order to account for the rapid turnover of calcium between blood and bone, in both directions, and for the ability of the organism to maintain constant calcium ion concentrations in the blood plasma, we have introduced the concept of a *dual mechanism.* The slow-acting part of this mechanism—that mediated by the parathyroid glands by control of osteoclastic resorption—is a clear example of a self-regulating process. The parathyroid glands are sensitive to the concentration of calcium ions in the blood; a deficiency in calcium ions leads to an increased activity of the glands, while an excess leads to decreased activity (Fig. 24). Such self-regulating processes have acquired the name *feedback.* The condition that is being regulated is itself the stimulus activating the regulatory mechanism; information about the output is fed back to an earlier stage so as to influence its action and thereby control the output. The living organism is dependent upon a multitude of such built-in feedback mechanisms. In this instance, since an excess of Ca^{++} leads to reduction in output, the term *negative feedback* is applied. The control normally maintains the plasma calcium level at approximately 10 mg. per 100 cc.; when the parathyroids have been removed the mechanism for release of calcium into the blood is absent; when there is a hyperfunctioning adenoma there is no evidence that it responds to the increased calcium ion concentra-

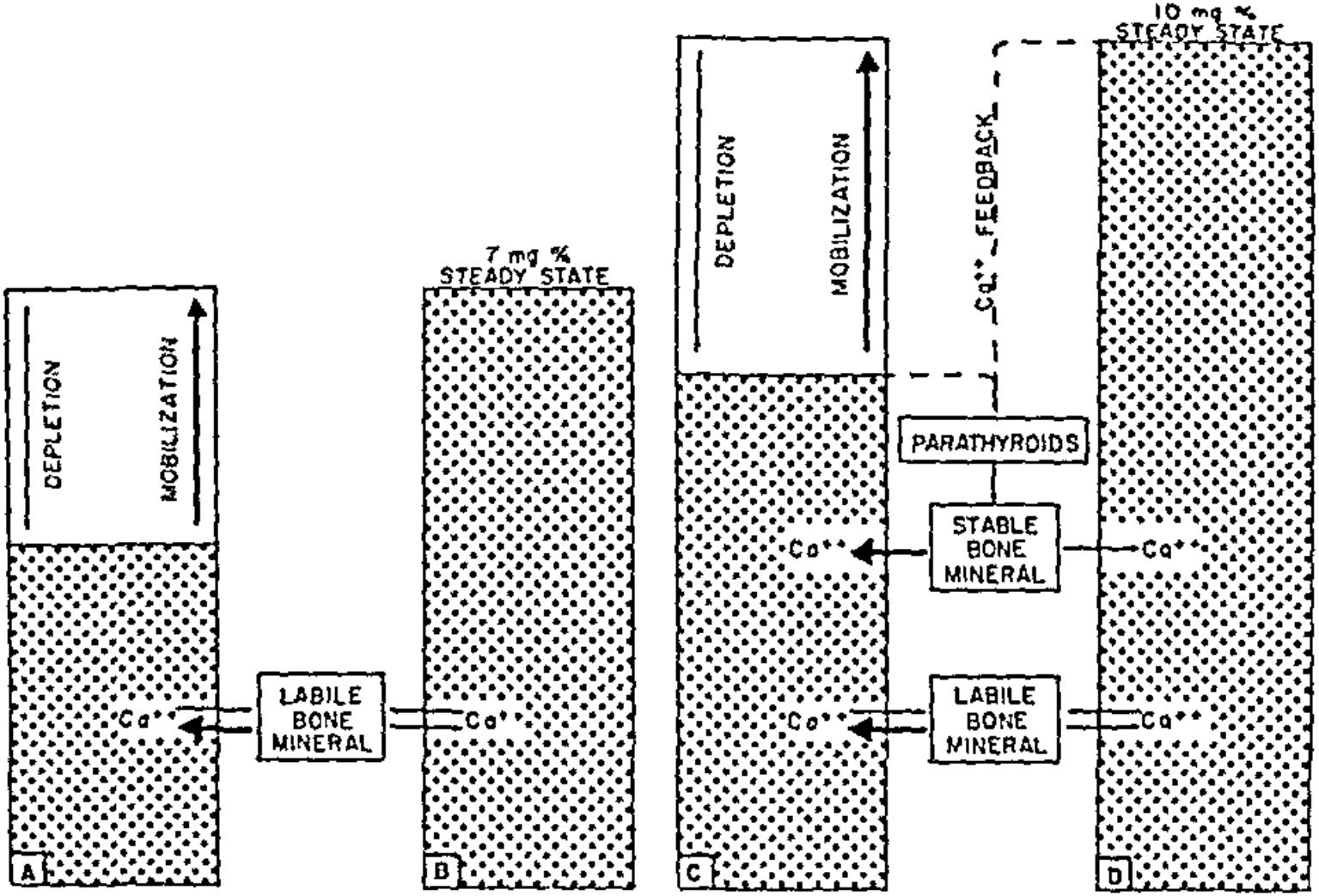


FIG. 24.—*Dual mechanism for homeostatic control of calcium ion concentration in the plasma. A*, restitution of calcium concentration in plasma *following* depletion, in the absence of the parathyroid glands. *B, steady* state as a result of movement of calcium to *blood; calcium* concentration is maintained at a level of approximately 7 *mg*/100 ml by equilibrium with labile fraction of bone *mineral. C*, restitution of calcium concentration in plasma in *presence of* parathyroid glands, by combination of diffusion from *labile fraction* and resorption of stable fraction of bone mineral. *D, steady* state at a level of approximately 10 mg/100 ml.

tion in the plasma; it appears rather that the regulatory system goes out of control.

The rapidly acting part of the dual mechanism requires ion transfer between blood and bone and is able in the absence of the parathyroid glands to maintain the plasma level of calcium at approximately 7 mg. per cent. One view is that the bone acts as an ion exchange column, taking up calcium ions from the blood and returning others, by passive physicochemical mechanisms. According to this, the labile or reactive bone mineral, or that in the newly formed and partially mineralized osteons, is in equilibrium with the ions in the fluid in contact with them. If the equilibrium is disturbed by removal of calcium ions from the blood, additional ions are transferred from the labile stores until the physiologic level has again been reached; if calcium is added to the blood, the excess is promptly transferred to the labile stores. This is, of course, subject to the conditions that the first line of defense, in either direction, is the extravascular fluid, and that diffusion through this fluid must occur before the pool of labile calcium in the bones is reached.

Another view is that this transfer mechanism may itself require intervention by cell activity, at least in the direction of bone to blood. Acceptance of this would require that the control be included within the feedback mechanism, since it is postulated that the parathyroid hormone acts to liberate calcium from the labile stores by increasing the production of acid, mainly lactic acid, by influencing the glycolytic cycle in the cells of bone. The role of acid production in the surface chemistry of the bone mineral has been reviewed in an earlier chapter. In any case, whether it is to be regarded as active or passive, the rapid transfer of calcium from the labile stores seems to be definitely localized in the new and incompletely calcified osteons.

On the other hand, if we regard the parathyroid hormone as regulating osteoclastic resorption of bone, by a direct action upon the osteoclasts or their precursor cells, it becomes clear that this part of the dual mechanism has access to a portion of the bone not accessible to passive ion transfer; Woods and Armstrong have pro-

vided direct evidence to support this statement. Osteoclasts are found only on the surfaces of bone and exert their effects upon resorption accordingly. The dual mechanism, then, affects the mobilization of calcium both from the labile fraction of bone, by ion exchange or transfer, and from the stable bone mineral, by resorption.

VITAMIN D AND CALCIUM HOMEOSTASIS

It has been indicated above that vitamin D plays a part in the homeostatic control of the calcium ion concentration in the blood; the mechanism of this action has not been fully clarified.

Vitamin D exerts an influence on the citrate content of bone; this effect has been attributed to a more rapid conversion of pyruvate to citrate in the tricarboxylic acid cycle. Vitamin D elevates the citrate content; vitamin D deficiency reduces it. Until recently, it was postulated that production of citrate was an important factor in the transfer of calcium from bone to blood, and that parathyroid hormone and vitamin D, although acting at different points in the glycolytic cycle, were synergistic in this respect. Now that the emphasis has been shifted to acid production in bone, and especially to lactic and pyruvic acids, and since acid production occurs before vitamin D is believed to exert its influence on glycolysis and on citrate production, the possible role of vitamin D is less clear.

This does not deny an effect of vitamin D on mobilization of bone mineral, even though it does not provide a mechanism for such an action. It has been suggested that this action of vitamin D is essential to calcium homeostasis, and that the maintenance of blood levels of serum calcium as high as 7 mg. per cent in the absence of the parathyroid glands depends upon this action. It was demonstrated, as early as 1930, however, that both dogs and rats can survive in the absence of both vitamin D and the parathyroid glands. There is no evidence that vitamin D liberates H^+ in glycolysis. For the present, although we accept the evidence for mobilization of calcium under the influence of vitamin D, the details of such an action remain obscure.

CHAPTER XI

Mineral Metabolism

1. Internal Environment of Bone

Bone tissue shares the internal environment with the other tissues of the body. This includes the circulating blood plasma and the intercellular fluid—a total of approximately 20 per cent of the weight of the body. An even larger amount of fluid, 50 per cent of the body weight, is intracellular; a small proportion of this is in the cellular elements of bone.

There is an extremely rapid exchange of water and dissolved substances between the various compartments of the body; this serves not only to bring oxygen to and take carbon dioxide away from the cells but also to transfer and exchange fluids and electrolytes. It is by the movement of water and dissolved substances that the minerals essential to the bones are brought to the locations at which they are to be deposited and are released when needed to maintain the physiologic constancy of the composition of the body fluids.

The important cation of the internal environment, from the standpoint of bone, is calcium. Of this element, a 70 kg. man has approximately 280 mg. in the circulating plasma, of which half, or 140 mg., is in the form of calcium ions. This does not exchange, to any considerable degree, with the contents of cells, which are virtually free from calcium, but it does exchange freely and rapidly with the 10 liters of intercellular fluid, which contains about 500 mg. of calcium, nearly all in the ionized form. As much as 100 per cent of the plasma calcium may exchange with that of the intercellular fluid and the bones every minute. The plasma plus the intercellular fluid forms the *calcium compartment*, amounting to

15–20 per cent of the weight of the body and containing, in a 70 kg. man, 525–700 mg. of calcium ions.

This amount of calcium, in the ionized form, is seemingly insignificant when compared with 1,200 gm. of calcium in the skeleton. Moreover, in view of the very rapid exchange of calcium between the calcium compartment and the bones, it is remarkable that the organism is able, by means of homeostatic mechanisms, to maintain a constant level of calcium ions in the body fluids, especially when an individual ion may stay in the plasma for only a matter of minutes.

Of the anions in the fluids of the body, the most important for the maintenance of osmotic pressure are Cl^- and HCO_3^-. From the *standpoint of the mineral constituents of bone, the most important* is inorganic phosphate, mainly as the ions $HPO_4^=$ and $H_2PO_4^-$. Since these ions exchange to some degree with the contents of cells, the space available to phosphate in the body is larger than the calcium space, but the same considerations with respect to the rapidity of exchange are valid also for phosphate ions.

The term *internal environment* was adopted by Claude Bernard to indicate that, as organisms become mobile and independent, they carry with them the constant and closely regulated environment necessary for the survival of their cells; the contrast is with minute organisms living in the environment of sea water. There is the very great difference that while the composition of the sea, as modified by exchange of its elements with the organisms living in this environment, changes relatively slowly, owing to the enormous volume of water, the internal environment is under the constant influence of rapid exchange with the cells. The net effect of such exchange is that the conditions of life for the cells are kept constant.

2. Homeostasis in Mineral Metabolism

Until recent years, it has been customary to regard the kidneys as functioning in an automatic fashion, with built-in mechanisms, responding to alterations in the composition of the blood plasma so as to conserve elements in short supply and to eliminate those in excess. More recently, it has become apparent that the ability

of the kidneys to act automatically is limited, and that increasing importance must be ascribed to central and hormonal control. We have advanced the hypothesis that the major cations of the blood plasma—Na^+, K^+, Ca^{++}, Mg^{++}, and H^+—are subject to such extrarenal control. In some cases the evidence is clear; in others it is only suggestive. On the whole the generalization seems permissible, at least as a working hypothesis, that the concentrations of all of these cations in the blood are centrally or hormonally controlled.

It is further proposed that for regulation of the cations of the blood plasma, the organism is supplied with a number of *information centers*, of which the respiratory center is one; each of these responds to information as to a particular state of affairs. The centers then relay this information, through a *chain of command*, to the organs whose function it is to apply the necessary corrections.

For the major anions of the blood plasma—Cl^-, HCO_3^-, $H_2PO_4^-$/$HPO_4^=$, and $SO_4^=$—the situation is quite different. As against the generalization that the cations of the blood are subject to central or hormonal regulation, the contrary statement may be made for the anions, i.e., that the control of none has been shown to depend upon information transmitted from a higher center, and that the existence of a feedback mechanism has been found for none.

With respect to homeostatic regulation of the cations of the blood, the major advance in recent years has been the discovery and partial elucidation of the control system which regulates the concentration of sodium in the body fluids. The system includes an extrahypophyseal diencephalic regulatory center, located in the hypothalamus, or perhaps in the region of the pineal body; this responds primarily to the level of serum electrolytes, either directly or by way of chemoreceptors. Neurosecretory cells of the regulatory center produce a tropic substance, called *aldosteronotropin* or *glomerulotropin*, which finds its way to the adrenal cortex, either directly by a humoral pathway or indirectly through the pituitary stalk and the adenohypophysis. This tropic substance, in turn, controls the output of *aldosterone*, which then influences the reabsorption of sodium by the renal tubules. This complex mecha-

nism fulfils all of the criteria for an information center, coupled with a chain of command that eventually leads to the corrections that must be made by the kidney. The kidney, while actually performing the functions that result in homeostasis, is far from doing so automatically, insofar as sodium is concerned.

Aldosterone, the sodium-retaining hormone elaborated by the adrenal cortex, is a *mineralo-corticoid*. Its properties are also exhibited by other adrenocorticoids, but aldosterone is many times more effective than is any other known natural steroid. The existence of a supraoptic information center, which controls the output of aldosterone, is well established, but many details of the control system remain in doubt.

We have shown above, that while calcium homeostasis is subject to hormonal control, it constitutes a special case, in that the parathyroid glands serve both as information centers and as the source of the hormone that controls the release of calcium from the bones to the blood. Homeostatic control of the calcium ion concentration in the plasma, mediated by the parathyroid glands, is also influenced by vitamin D.

3. Calcium

Calcium of Fluids of the Body

All or nearly all of the calcium in the blood is in solution in the plasma; the amount in the red blood cells is negligible. In normal man the concentration of calcium in the plasma is usually between 9 and 11 mg. per 100 cc., with 10 mg. per 100 cc. (2.5 mM per liter) a representative figure. This is in constant exchange with the calcium of the extracellular fluid and that of the bones; the homeostatic mechanism that maintains the constancy of the concentration in the plasma is the function of the parathyroid glands. In the absence of the parathyroid glands, the plasma calcium may fall to 7 mg. per 100 cc. or even lower. In clinical or artificially induced hyperparathyroidism the figure may reach a level of 15 mg. per 100 cc. or higher.

It has been known since early in the present century that the calcium in the plasma is separable into two major and roughly equal fractions—diffusible and non-diffusible. It has also been known

that the non-diffusible fraction is associated with the plasma protein. There was, however, for some years, a difference of opinion concerning the diffusible fraction. It was held by many that a major portion of this fraction is in the form of a complex with the citrate ion or with some other comparable substance. These uncertainties have been resolved, and the relationship of calcium to the plasma protein has been incorporated in a formulation which describes the plasma as a solution of a weak electrolyte, calcium proteinate, the ionization of which may be represented by the equation:

$$CaProt \rightleftarrows Ca^{++} + Prot^{=} \qquad (1)$$

and by the mass-law equation:

$$\frac{[Ca^{++}] \times [Prot^{=}]}{[CaProt]} = K_{CaProt} = 10^{-2.22}. \qquad (2)$$

From these two equations and from the expressions [Total Ca] = [CaProt] + [Ca^{++}], and [Total Prot] = [CaProt] + [$Prot^{=}$], there may be derived a general equation from which the calcium ion concentration of the plasma or serum may be calculated from analyses for total calcium and total protein—quantities easily determined in the laboratory:

$$[\text{Total Ca}] = \frac{[Ca^{++}] \times [\text{Total Prot}]}{[Ca^{++}] + K} + [Ca^{++}]. \qquad (3)$$

The calculation is facilitated by the use of a nomogram, constructed from equation 3, shown in Figure 25, which illustrates the relationships of the ionized and unionized fractions of calcium at varying protein and calcium levels.

At normal protein levels, about half the plasma calcium is in the ionized form, the other half being mainly in an undissociated complex with protein. Since this is an ionization phenomenon, shifts from the ionized to the unionized form occur instantaneously; the unionized calcium is not in firm combination with protein. Moreover, since rapid movement of ions occurs throughout the organism, the formulation does not describe a static system; it does describe a system in dynamic equilibrium.

According to the mass-law equation 2, the ratio Ca^{++}/CaProt is determined by the concentration of protein. When the total calcium falls, as in hypoparathyroidism, or when it rises, as in hyperparathyroidism, the ratio of ionized to unionized calcium should remain approximately constant, with only a small correction for a shift in the CaProt/$Prot^{-}$ ratio, unless the concentration of protein changes or there is some qualitative change in the ability of the protein to complex calcium. That the relationship predicted by the mass-law equation is approximated over the entire range in serum calcium levels from hypoparathyroidism to hyperparathyroidism was reported by McLean, Barnes, and Hastings, in 1935, their cal-

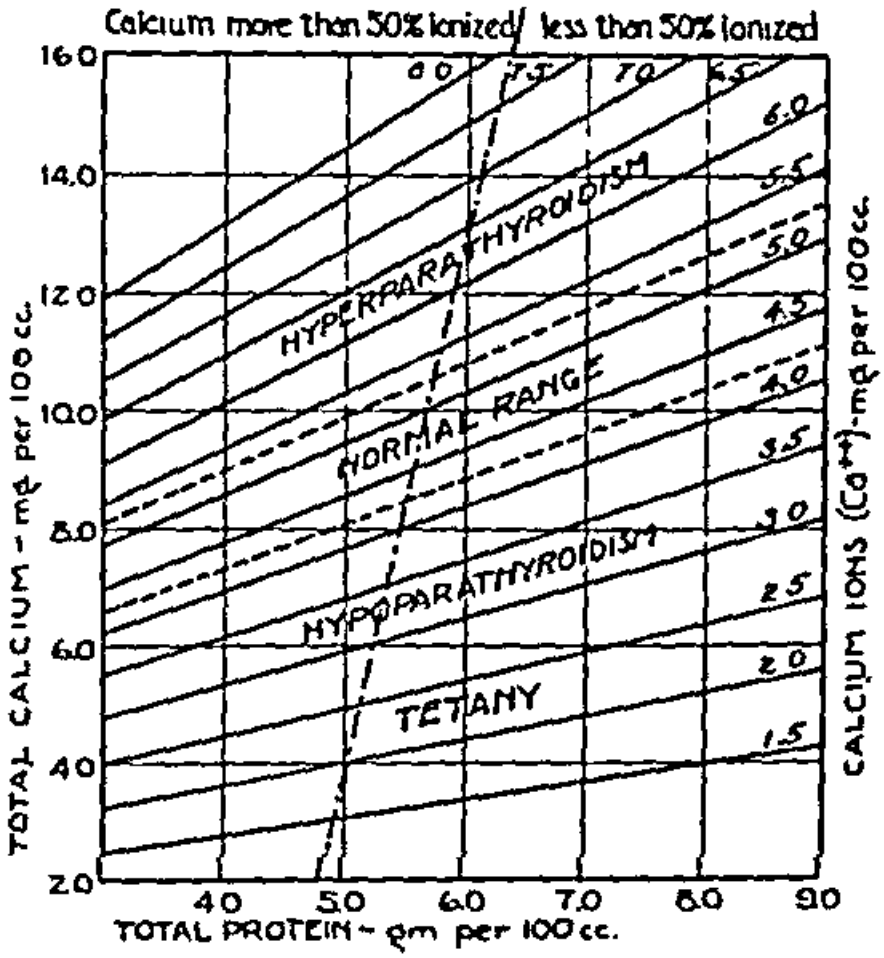


Fig. 23.—Nomogram for calculation of Ca^{++} concentration from total protein and total calcium of serum or plasma. Constructed from mass-law equation $[Ca^{++}] \times [Prot^{-}]/[CaProt] = K$. *(From Fig. 2, McLean and Hastings, Am. J. M. Sc., 189: 606. Reproduced by courtesy of the publishers.)*

culations being based on direct observation of Ca^{++} concentrations by the frog-heart method.

Freeman and his co-workers, using the ultracentrifuge to separate protein-bound and free fractions of plasma calcium, and Rose *et al.*, using ultrafiltration for the same purpose, have found that while the amount of calcium complexed by protein is correlated with changes in the total calcium concentration, the quantitative results have not conformed to the simple mass-law equation 2; the discrepancies are attributed by Rose to an influence of the parathyroid hormone on the affinity of the serum proteins for calcium, while Freeman believes that they are correlated with the serum calcium concentration, and only indirectly with parathyroid activity.

Others have *reported further discordant and still unexplained* results. In our opinion the more recent findings, with different methods, do not invalidate the concept of calcium proteinate as a weak electrolyte, dissociating in accordance with a mass-law relationship; further refinement of methods, and further attention to variables other than total calcium, total protein, and calcium ions, seem to be required for resolution of the differences in observations and interpretations.

Differences in the affinity of serum albumin and serum globulin for calcium have also been reported, although current literature supports the view that these proteins, in the serum of normal individuals, have approximately the same calcium-binding capacity. This statement does not hold true for certain forms of hyperglobulinemia. That serum albumin, in normal blood, combines with more calcium than does serum globulin depends upon the higher concentration of albumin in the plasma.

This description, which takes into account only calcium ions and the unionized calcium proteinate fraction, ignores a small amount of calcium in other combinations. Calcium forms unionized fractions with the ions of phosphate, bicarbonate, and citrate, but the aggregate of all unionized complexes in the blood, with the exception of proteinate, can hardly account for more than 5–10 per cent of the total calcium. When serum is subjected to diffusion or to ultrafiltration, these fractions appear in the diffusible portion. For

this reason the total diffusible calcium is slightly higher than the calcium ion concentration. Since the concentration of citrate in the blood is correlated with parathyroid activity, the calcium-citrate complex may account for a part of the discrepancies observed in the calcium-protein relationship.

In the protein-poor fluids of the body, such as the cerebrospinal and the intercellular fluids, the state of calcium approximates that in an ultrafiltrate of plasma. In such fluids the concentration of calcium is generally about 5 mg. per 100 cc. (1.25 mM per liter), virtually all in the diffusible form. Again, a fraction of this may be in the form of unionized, diffusible complexes; these have little or no physiologic significance.

In calcification, when calcium is to be deposited in the bones, it *moves first from the plasma to the intercellular fluid and then is* deposited in locations prepared for calcification. Under such circumstances the plasma requires replenishment, in order to maintain calcium ion concentrations at physiologic levels; such replenishment ordinarily comes from the diet, but the skeleton itself is the principal depot for the storage of reserve supplies in the organism. Thus, it frequently comes about that as calcium is being deposited in one or more places in the skeleton, it is being withdrawn from other places; the entire system *plasma-intercellular fluid-bone* remains in a state of dynamic equilibrium.

4. Physiology of Calcium Metabolism

Requirements

The requirements of calcium, under varying conditions, have been the subject of much study. The Recommended Dietary Allowances, as revised by the Food and Nutrition Board, National Academy of Sciences–National Research Council, in 1958, are given in Table 2.

All these recommended allowances are well above minimum maintenance levels and provide for a liberal margin of safety. Except for the special conditions of pregnancy and lactation, when calcium should be added to the diet, the requirements are such that they are met or exceeded by the ingestion of one quart of milk

daily, if the diet is otherwise adequate. Many adults who do not drink milk are able to maintain themselves in calcium balance with intakes of calcium at 10 mg. per kg. per day, or 0.5–0.8 gm. daily.

ABSORPTION AND EXCRETION

The absorption of calcium takes place chiefly in the upper part of the small intestine, in both the duodenum and the jejunum. A large and variable part of the ingested calcium passes through the alimentary tract unabsorbed. Moreover, a considerable quantity of calcium, having been absorbed, is secreted with the digestive

TABLE 2

	Gm
Adults (male and female)	0 8
Pregnancy (latter half)	1.5
Lactation (28 oz. daily)	2 0
Infants, 2–6 months	0 6
7–12 months	0 8
Children, up to 12 years	
1–3 years	1 0
4–6 years	1.0
7–9 years	1 0
10–12 years	1.2
Children, over 12 years	
Girls	
13–15 years	1 3
16–19 years	1 3
Boys	
13–15 years	1.4
16–19 years	1.4

juices; the greater part of this is reabsorbed; a smaller portion appears in the feces. From the standpoint of *net absorption*, normal adults may utilize as little as 20 per cent of the calcium ingested. In *rickets* the net absorption is greatly reduced and may even fall to negative values, indicating a loss rather than a gain.

The most important single factor in the absorption of calcium is vitamin D. Absorption of calcium is unfavorably influenced by *oxalate* and *phytate* ions, and by failure of absorption of fatty acids from the intestinal contents, in the condition known as *steatorrhea;* in each case calcium passes through to the feces in an insoluble, non-absorbable form. There is a widespread belief that acids and acidifying substances influence the absorption of calcium favorably

and alkaline substances unfavorably. Experimental evidence is to the effect that such influences are too small to be of physiologic significance. The calcium intake markedly influences the net absorption of phosphate from the alimentary tract; the corresponding influence of phosphate upon the net absorption of calcium is negligible.

Loss of calcium from the body takes place through two channels, the alimentary canal and the urinary tract. Under ordinary conditions the calcium in the feces is greater in amount than that of the urine and under conditions of low intake may exceed that in the food. It is no longer believed that there is any regulated excretion of calcium into the intestinal contents. The effect of increased ingestion of calcium upon net absorption is variable. Most healthy adults are able to adapt to low intakes of calcium, provided that there is no deficiency of vitamin D. Moreover, while extremely low intake of calcium (less than 200 mg. daily) may be presumed to lead to continuous negative balances, there is no agreement as to when this results in an unphysiologic condition, so long as the plasma calcium is maintained within normal limits In infants the absorption and utilization of added calcium is, within wide limits, proportional to the intake. Calcium administered parenterally or mobilized from the skeleton by parathyroid extract appears in the urine to the extent of approximately 80 per cent of that excreted; the remaining 20 per cent appears in the feces.

In adults the utilization of calcium is judged by the difference between ingested and fecal calcium. In infants and growing children the term is used in the sense of retention in the organism to meet the needs of growth, particularly of the skeletal system.

There is no food or drug that yields calcium as freely as milk. Calcium is in milk in what may be called a physiologic solution, together with phosphorus and with the protein to be utilized for the building of bone tissue. For these reasons milk is generally more effective than calcium phosphate or lactate or than any other single substance in providing for the needs of growth or repair of bone—even more effective than bone meal itself. The calcium of breast milk is no better utilized than that of cow's milk. Evaporated or

dried milk is a satisfactory substitute for whole milk in promoting retention of calcium and the formation of bones in children. The utilization of dietary calcium varies inversely with the adequacy of the body's stores.

BALANCE

The calcium balance represents the net excess or deficit of retention of the element, as compared with the intake. The normal condition for a growing child is a substantial positive calcium balance, representing the retention necessary for skeletal growth. A daily storage of 10 mg. per kg. of body weight in children from three to thirteen years of age is a desirable condition. For the adult the normal condition is calcium balance; a large proportion of the adult population is usually in negative balance, owing to insufficient intake. Negative calcium balances may be observed in women during early lactation, in spite of large intakes.

Calcium balance studies have been useful in the clinic. They have revealed a negative balance in patients with hyperparathyroidism, at stages and in forms of the disease in which there were no bone changes demonstrable by X ray; they are informative in clinical investigations in which it is necessary to determine whether therapy is effective without having to wait for gross and unequivocal evidence of improvement. An outstanding example is the discovery that a mixture of citric acid and sodium citrate will spare serious loss of calcium in patients with certain types of acidosis.

KINETICS OF CALCIUM METABOLISM

With the aid of radioisotopes there are increasingly frequent attempts to define the kinetics of the movement of the ions concerned in the homeostasis of their concentrations in the fluids of the body and in the mineralization of calcifiable tissues. Such attempts began with the construction of curves illustrating the disappearance from the blood of an isotope, now commonly Ca^{45}, following its intravenous administration. These curves were then subjected to kinetic analysis and expressed as a composite of exponential decay curves. From these data there were calculated the turnover rates of the calcium ions in the blood, with the result that in young ani-

mals this rate may be as high as 100 per cent of the total amount in the blood per minute. In adult man every fourth calcium ion leaves the blood every minute.

A wide field was opened for investigation by further extension of tracer techniques when, with the aid of computations based on blood disappearance curves and on observations of uptake of Ca^{45} by the bones of experimental animals, it became possible to calculate the rates of accretion, resorption, and exchange reactions in the skeleton—parameters previously inaccessible to analysis At present most workers in this field base their analyses, with or without modifications, on an approach published in 1955 by Bauer, Carlsson, and Lindquist and further elaborated in subsequent papers from their laboratories. Their method depends upon observations made after injection of a single dose of the radioisotope, and upon the following equations:

$$Ca^{45}_{Obs} = Ca^{45}_{E} + Ca^{45}_{A} - Ca^{45}_{R} \qquad (1)$$

in which

Ca^{45}_{Obs} = total amount of Ca^{45} present in a calcified tissue

Ca^{45}_{E} = amount of Ca^{45} present in the exchangeable fraction of the bone salt

Ca^{45}_{A} = amount of Ca^{45} incorporated into the non-exchangeable fraction of the bone salt through accretion

Ca^{45}_{R} = amount of Ca^{45} removed through resorption.

Under the assumption that the specific activity $Ca^{45}/(Ca^{40} + Ca^{45})$ of the exchangeable fraction rapidly attains the same value as the specific activity of the plasma Ca, and that these two specific activities can then be regarded as equal, we get:

$$Ca^{45}_{F} = E \times S \qquad (2)$$

in which

E = amount of total Ca in the exchangeable fraction of the bone salt

S = specific activity of the serum (plasma) Ca.

Under the assumption that Ca^{45} and Ca^{40} are deposited in the bone mineral in the same proportions as those in which they are

present in the plasma at any instant of time, there is derived:

$$Ca_A^{45}(t) = A \int_0^t S(t)\,dt \qquad (3)$$

in which

A = rate of accretion of total Ca

t = interval of time between administration of Ca^{45} and observation

$\int_0^t S(t)\,dt$ = integrated specific activity of serum from time *0* to time *t*.

The rate of resorption of total calcium is calculated from the equation:

$$R = A - Ret \qquad (4)$$

in which

R = rate of resorption of total calcium

Ret = rate of change in total calcium in bone.

From these formulations, and by further analysis of data obtained by counting methods, with the solving of simultaneous equations, values are obtained for the rates of accretion (A) and resorption (R) and for the amount of calcium in the exchangeable fraction of the bone mineral (E). In the normal adult new bone mineral is formed at a rate of about 0.5 gm. calcium per day, and a corresponding amount is resorbed. The adult skeleton contains between *1,000* and *1,500* gm. calcium, and is thus renewed at a rate of about 0.05 per cent per day. In the newborn the corresponding rate is 1 per cent or higher. Some caution must be exercised in the application of these turnover values, since the rate of renewal varies considerably from area to area in the skeleton.

By the use of Ca^{45} and similar, though not identical, formulations and assumptions, Heaney and Whedon have reported on the bone formation rate in human metabolic bone disease. From their own observations, and from data in the literature, they have estimated the normal rate of formation of bone mineral, in the human adult, to be approximately 9 mg Ca/kg/day, with a mean in ten subjects of 9.1 ± 3.9 S.D. In twelve adult human subjects, with or without bone disease, the size of the miscible calcium pool averaged

slightly less than 100 mg Ca/kg in all subjects, except for two patients with osteitis deformans, in whom the pools were four times as large. The rates of bone formation in six patients with osteoporosis were within the normal range. A reduced rate of bone formation was found in one patient with hypoparathyroidism, and greatly elevated rates were observed in two patients with osteitis deformans.

TABLE 3

CALCIUM ACCRETION IN BONE DISEASE

High
- Paget's disease
- Fracture
- Tumor
- Hyperparathyroidism
- Hyperthyroidism
- Vitamin D–resistant rickets treated with massive doses of vitamin D

Normal
- Osteopenia (osteoporosis) of unknown origin
- Vitamin D–resistant rickets
- Vitamin D–deficient rickets after treatment with vitamin D

Low
- Osteopenia of unknown origin
- Hypoparathyroidism
- Hypothyroidism
- Vitamin D–deficient rickets

A further advance, especially in the observation of patients, has been made possible by the use of γ-emitting isotopes of calcium (Ca^{47}, half-life 4.9 days) and strontium (Sr^{85}, half-life 65 days) which permit tracing the injected isotope by means of scintillation counters at the body surfaces. Such studies in man show the rapid rise and fall in soft tissue activity, and the slow rise in bone activity. The skeleton does not distinguish between calcium and strontium in trace amounts; the results obtained from external counting over various lesions following administration of Ca^{47} or Sr^{85} may therefore be discussed without distinction between the two elements. Localized bone lesions in man are particularly easy to study with external counting techniques.

Wendeberg has demonstrated in fifty human subjects that following fracture of the tibial shaft the rate of bone salt formation in the fracture area is rapidly increased to a peak value of about twenty times normal, and then gradually falls; the peak value is reached at about one-half to one year following the fracture. The rate of turnover of bone mineral is also increased in localized bone lesions caused by tumor, osteitis, and Paget's disease; in a few cases the use of external counting techniques permitted a diagnosis of metastatic cancer in the spine before X-ray evidence was positive.

Bauer has summarized the findings from several laboratories, obtained by external counting of γ-radiation from Ca^{47} and from Sr^{85}, in patients with bone disease, as shown in Table 3. These observations are leading to revision of current concepts of the pathologic physiology of bone; they indicate, for example, that the accretion rates in osteoporosis are normal or only slightly depressed, contrary to earlier views.

5. Phosphorus

Phosphates of fluids of the body

Inorganic phosphate is present in the fluids of the body in the form of the ions of orthophosphoric acid, H_3PO_4, chiefly as $HPO_4^{=}$. The plasma of the infant, under conditions of active deposition of bone salt, contains approximately 6 mg. per 100 cc. of total phosphorus as inorganic phosphate (2 mM per liter). In the plasma of the adult the inorganic phosphate level is reduced to approximately half that of the infant It is characteristic of the rachitic infant that the concentration of inorganic phosphate in the plasma is at a level normal for the adult. The excretion of phosphate through the kidneys varies within wide limits, according to the intake; this has the effect of maintaining the blood phosphate at a relatively constant level; the part played by the parathyroid hormone in homeostatic *regulation of the blood level of phosphate* is imperfectly understood.

The transport of phosphate within the animal organism is a function of the *circulating blood and of the intercellular fluid.* Since the phosphates have a manifold role in the organism and are closely related to many metabolic functions, as well as to calcification, they are found in many forms and in many locations. In the

blood, in addition to the inorganic phosphate of the plasma, a variety of organic, acid-soluble compounds of phosphorus is present, mainly in the red blood cells; only about 0.5 mg. per 100 cc. of phosphorus is carried in the plasma as phosphoric esters. The movement of phosphate within and between the fluid compartments of the body and between fluid and bone occurs constantly and with the same rapidity as that with which the movement of calcium occurs.

So far as is known, the inorganic phosphate of the fluids of the body of higher vertebrates is virtually all in the form of free ions. Various attempts have been made to establish the presence of phosphate in a bound form, analogous to the unionized fraction of calcium. There seems, at present, to be no reason to accept a working hypothesis that accounts for any physiologically significant fraction of the inorganic phosphate of the blood as being present in an unionized complex.

METABOLISM OF PHOSPHORUS

An examination of the multiple role of phosphorus in the animal organism is beyond the scope of this volume. Except for a brief recapitulation of the types of phosphorus compounds found in the organism, this section will be concerned with the relationship of the metabolism of phosphorus to the skeleton.

All or almost all the compounds of phosphorus, inorganic or organic, found in the body are derivatives of orthophosphoric acid, H_3PO_4, or of pyrophosphoric acid, $H_4P_2O_7$, itself a condensation of two molecules of orthophosphoric acid.

Of approximately 700 gm. of phosphorus in the adult human body, about 600 gm. are in the skeleton; the presence of this large amount of phosphorus in the bones depends upon the ability of orthophosphoric acid to form difficultly soluble compounds with calcium.

Orthophosphoric acid is a weak tribasic acid. Its dissociation constants, corrected for the ionic strength of serum, are given in Table 4. From these constants it may be calculated that, at pH 7.4, approximately 85 per cent of the total inorganic phosphate of the

plasma is in the form of the divalent ion $HPO_4^=$, while 15 per cent is monovalent $H_2PO_4^-$, and only 0.0035 per cent is trivalent $PO_4^{\equiv}$.

A major factor in the metabolism of phosphorus is that the organism is able to synthesize all of the organic compounds of phosphorus from inorganic phosphate. This is not to say that it can synthesize all of the organic substances with which phosphate is united. The human organism, for example, cannot synthesize thiamine. But given thiamine, the organism can transfer it into the *active form, thiamine pyrophosphate. Moreover,* phosphate ingested in organic combination is for the most part split off and absorbed in the same manner as if ingested as inorganic phosphate. The biosynthesis of the large number of phosphorus-containing

TABLE 4

DISSOCIATION OF H_3PO_4

$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^-$,	$K_1' = 1.22 \times 10^{-2}$,	$pK_1' = 1.915$,
$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^=$,	$K_2' = 2.19 \times 10^{-7}$,	$pK_2' = 6.66$,
$HPO_4^= \rightleftharpoons H^+ + PO_4^{\equiv}$,	$K_3' = 1.66 \times 10^{-12}$,	$pK_3' = 11.78$.

catalysts and intermediates requires additional enzyme systems, many of which themselves contain phosphorus. Phosphorus provides the linkage between the nucleotides which compose ribose and deoxyribose nucleic acid and thus contributes to the structure of chromosomes and the processes of growth and heredity. It participates in the formation of numerous intermediate compounds and coenzymes essential to the metabolism of carbohydrates, as well as to many oxidation-reduction reactions and other intracellular processes.

Until recently *pyrophosphate* had been found in mammalian tissues in only very small amounts, in such organic compounds as thiamine pyrophosphate. Cartier and Picard, however, found that when phosphate was supplied to embryonic sheep cartilage in the form of adenosine triphosphate, approximately 80 per cent of the phosphate deposited in the cartilage was pyrophosphate. The corresponding figure for rachitic rat cartilage was found by Perkins and Walker to be only about 5 per cent; they attributed the dis-

crepancy to a difference in the inorganic pyrophosphatase activity of the two tissues. Perkins and Walker also reported, for the first time, the finding of pyrophosphate in normal bone and found that the deposit formed *in vitro* on incubation of rachitic cartilage with a calcifying medium does not contain pyrophosphate unless ATP is added to the substrate, when the proportion of pyrophosphate attains the same order as that in normal bone. These findings seem to relate ATP, which has been called the *unit of currency* in metabolic energy transformations, and pyrophosphate to the calcification process, although the nature of this relationship is by no means clear. No one has as yet shown that energy, supplied locally, is essential to the deposition of bone mineral, although this possibility is receiving increased attention. If this could be demonstrated, the presence of ATP in calcifying systems would assume new significance.

SOURCE, REQUIREMENTS, AND ABSORPTION

As phosphorus is present nearly everywhere in the animal organism, so is it present nearly everywhere in nature. Ultimately, the source of the phosphorus in the animal is the sea or the soil. For marine plants and animals, phosphorus is always in short supply, 40 μgm. per liter of sea water; this small concentration must be turned over very rapidly to support life. Indeed, the population of living organisms in aquatic habitats appears to be limited as much by the supply of phosphorus as by any other single element of nutrition. For man the flow is first to the plant foods, then either direct to the human organism or indirectly by way of the animal tissues consumed by man. Fertilization of the soil consists in supplying it with phosphorus, as well as with nitrogen—two elements without which most plants cannot grow. A deficiency of phosphorus in the soil may limit plant and animal life.

The daily requirement for phosphorus in the adult human organism is in the neighborhood of 1 gm. and is even higher in the growing child, in whom the needs of the skeleton must be met. In American diets phosphorus is so universal that a deficiency in the intake of this element scarcely constitutes a problem in nutrition. If the dietary requirement for calcium is met from milk, the phos-

phorus intake will be adequate, as it will be from other protein-rich foods. Certain borderline diets are deficient in phosphorus, but the importance of this is overshadowed by other concomitant deficiencies The phosphorus content of mother's milk is much lower than that of cow's milk and may, in breast-fed infants, be the limiting factor in the rapidity of mineralization of the skeleton. This cannot be regarded as a true deficiency. A deficient absorption or utilization of phosphate, in the presence of an adequate intake, is commonly associated with the low retention of calcium in rickets. The total effect is as though there were an actual deficiency of both calcium and phosphorus in the diet.

In view of the competing demands for phosphorus to serve its manifold functions in the organism, it is important to note that the first effect of a deficient assimilation of phosphorus is a failure of calcification of the bones and that this failure, by removing the largest requirement for the element, usually leaves an amount adequate for all other purposes, except under the most extreme experimental conditions.

COLLOIDAL CALCIUM PHOSPHATE

Increase in the product Ca × P to a point beyond the solubility product constant of $CaHPO_4$ not only is critical for deposition of the bone mineral but also determines the formation of a colloidal calcium phosphate in the plasma. This substance, although in particulate form, is not precipitated from the blood, owing to the protective action of the plasma proteins. It is quickly removed from the blood by the histiocytes of the liver and spleen and is returned to the circulation in solution. It has not been shown that the formation and disposal of this substance are of physiologic importance or that it plays any part in the formation of the bone salt.

6. Bone as Reservoir of Minerals

The bones serve as a reservoir of calcium and phosphate, available for the other needs of the body, as well as for supplying minerals for deposition when needed in other parts of the skeleton. Moreover, other mineral elements may be deposited in bone and may remain there throughout the life of the organism. In so doing, as a rule they serve no known physiologic function, although sodium

and magnesium may be mobilized when needed by the soft tissues. In growing animals the bones respond to changes in intake of calcium and phosphorus, either by reducing or by increasing the amount of spongy bone available for storage. In cases of extreme deficiencies, either of calcium or phosphorus or of vitamin D, profound changes in the state of mineralization of the bones may occur.

EFFECTS OF INTAKE OF CALCIUM AND PHOSPHORUS UPON BONE

Sherman and his co-workers investigated the calcium requirements of the laboratory rat and introduced the concepts of *adequate* and *optimum* diets. An adequate diet is one that will support normal growth, health, reproduction, and lactation, generation after generation; an optimum diet will produce increased growth, earlier maturity, higher adult vitality as indicated by superior breeding records, a longer period between the attainment of maturity and the onset of senility, and an increase in the average length of adult life. These concepts have been useful in guiding experimental work and in interpreting the conflicting results in the literature concerning the calcium requirements of man.

The bones of rats on diets classified as adequate in calcium intake may be seen to have: (1) a relatively short metaphysis and therefore a relatively short supply of stored calcium; (2) great osteoblastic and osteoclastic activity, reflecting the need for maximum turnover and utilization of the dietary calcium; (3) osteoid borders on the trabeculae, indicating that calcium is not being stored to the maximum capacity of the bone structure; and (4) a porous shaft, similar to that of very young animals.

The bones of rats on diets classified as optimum, on the other hand, have: (1) a relatively long and dense metaphysis, providing for the maximum storage of calcium; (2) diminished osteoblastic and osteoclastic activity, indicating minimum turnover and maximum conservation of bone salt; (3) bone trabeculae calcified to the maximum density with no osteoid borders, evidence of an abundant and continuous supply of calcium and phosphorus in the diet and body fluids; and (4) a very dense shaft similar to that of adult animals.

CITRATE

It has been known, since the report of Dickens in 1941, that 90 per cent or more of the citric acid of the body is in the skeleton and that as much as 1 per cent of the fresh weight of bone may be accounted for as citrate.

The occurrence of citrate in the mineral portion of the skeleton forms one of the strongest arguments for the belief that such substances are held on the surfaces of apatite crystals, since the size of the citrate molecule clearly excludes it from the apatite structure itself. Whether citrate is present in bone as the citrate ion or whether it is combined in a complex form with calcium is still not clear. That portions of the citrate may be dissolved from powdered bone only with difficulty, while other portions are readily soluble, suggests either that more than one form of citrate exists in bone or that portions of the citrate are held on entrapped surfaces.

Present interest in citrate centers upon its functional significance in bone. It is probable that at least part of the citrate in the circulating blood originates in the skeleton and is metabolized by the kidney. Moreover, the increase in the citrate in bone and in the blood, as induced by either the parathyroid hormone or vitamin D, or both acting together, offers presumptive evidence that citrate plays an important role in the solubilization of the bone mineral and the transfer of calcium from bone to blood. It is not yet clear, however, how citrate is related to calcium metabolism; Neuman now puts more emphasis on the production of acid in the glycolytic cycle, with a consequent increase in the local concentration of hydrogen ions, than on the complexing of calcium by the citrate ion. Consideration has been given to these problems in an earlier chapter.

STORAGE OF CALCIUM AND PHOSPHATE

Except for the secondary system of medullary bone formed in birds, during the egg-laying cycle, such storage of calcium and phosphate as occurs in the skeletal system is limited to the bones that also have a supporting function. There is, in mammals, no special storage mechanism to meet the needs of pregnancy and lac-

tation; skeletal calcium and phosphorus are, however, readily given up to meet the needs of the soft tissues. This enables homeostatic regulation of a normal calcium level in the plasma to be maintained over long periods of time, at the expense of a negative calcium balance; it also gives the manifold requirements of phosphate in the soft tissues a priority for the assimilation of the phosphate in the diet. Except under extreme conditions, e.g., in the Middle European countries in World War I, and in China, where the drain of pregnancy on the skeleton, coupled with a deficient intake of calcium and of vitamin D, may lead to severe osteomalacia, the organism is able to adapt to a low intake of calcium without demonstrable pathologic changes. The possible relation of calcium deficiency to osteoporosis is discussed in a later chapter.

STORAGE OF OTHER ELEMENTS

In addition to calcium, the skeleton contains other cations, some of which are foreign to the needs of the organism and have been ingested fortuitously. Of the additional cations found in the bone mineral, two are of physiologic importance, and for these the skeleton may be regarded as a storage reservoir, aiding in the maintenance of physiologic levels of these elements—sodium and magnesium—in the blood, in the event of depletion. Potassium has no special affinity for bone.

A number of foreign cations are found as contaminants of the bone mineral, having been deposited after reaching the blood stream. Of these the most important are radium, strontium, and lead, any of which may substitute for calcium in the crystal structure of hydroxyapatite. Much recent attention has been directed to the harmful effects of radioactive elements when incorporated within bone; this is given consideration in the following chapter.

SODIUM

Some 46 per cent of the total body sodium is present in the mineral substance of bone, at a concentration approximating 400 mEq/kg of bone salt. The effective concentration of sodium at the crystal surface is probably higher. Some 40–45 per cent of the total sodium of the skeleton is rapidly exchangeable, and available to

the fluids of the body; an additional 6–13 per cent can be mobilized in the adult, in response to various stimuli. The ability of bone to release sodium ions to protect the hydrogen ion concentration of the extracellular fluids, in acute sodium depletion, appears to be dependent upon parathyroid secretion. The portion of the bone sodium not accessible to the body fluids is associated with the stable fraction of the bone mineral. Bone can also serve as an acceptor of Na^+, up to 8 per cent of the normal crystal content.

Vincent has studied sodium metabolism in the skeleton at the histologic level, employing two isotopic methods. He has induced radioactivity in ground sections of bone, by exposure to a neutron flux, which activates Na^{23} by forming the radioisotope Na^{24}, with a half-life of 15.06 hours; he has also administered Na^{22}, with a half-life of 2.6 years; in both instances sections of bone were studied autoradiographically and by means of microradiograms.

Bauer gave Na^{22} to growing rats, and killed them 1–25 days later. By means of autoradiograms he showed that during growth sodium is incorporated in the non-exchangeable fraction of the bone sodium, from which it is not removed until the bone structure is reached by resorption. On the assumption that the sodium of the extracellular water of bone is freely exchangeable with the serum sodium, and in another series of experiments with Na^{22}, he concluded that 30–40 per cent of the excess sodium of bone is exchangeable with the extracellular water sodium. Vincent, in short-term experiments, in which the dynamics of sodium deposition in bone was studied after administration of Na^{22}, found that rapid exchange mechanisms predominate; no accumulation of sodium was observed either in the sites of osteogenesis or in the less calcified osteons. Radioactivity disappeared on decalcification but was retained in ethylenediamine-treated sections; the sodium was linked to the mineral of bone. In longer term experiments, four to six weeks following injection of Na^{22}, there was concentration of the radioisotope in osteons in which bone mineral was being deposited at the time of injection; this is evidence for accretion of new sodium during initial mineralization. After immersion in a solution of $Na^{22}Cl$, compact bone is uniformly labeled.

MAGNESIUM

Magnesium is believed to be chemisorbed on the surface of the crystals of bone mineral and is readily available to the fluids of the body, at least in large part; the complex $MgOH^+$ has also been proposed as a surface ion. No homeostatic mechanism for regulation of the exchange of magnesium between blood and bone is known. About a third of the skeletal magnesium can be mobilized in severe magnesium deficiency; this is accomplished by ion transfer from the labile stores in the skeleton to the circulating fluids.

CHAPTER XII

Radiation, Isotopes, and Bone

Neither radiation itself nor the metabolism of radioactive isotopes has any place in the normal physiology of bone. Within the last half-century, however, and more especially in the last two decades, these subjects have assumed increasing importance, with the skeleton playing a major role in relation to them. Excessive doses of X ray to the bones, particularly during growth, may be harmful, and these effects have been studied experimentally. Early studies of the effects of internal radiation of the bones were confined to the damage caused by radium. Accidental poisoning by radium, usually resulting from exposure some years ago, especially in radium-dial workers and in patients given radium therapeutically, continues to receive attention. The occurrence of osteogenic sarcoma was attributed to radium as early as 1929.

The introduction of the *cyclotron*, the subsequent development of *nuclear reactors*, and the exploitation of these and other instruments for the production of radioactive isotopes and even of new elements have increased both the hazards of radiation and the opportunities for the study of its effects. Concurrently, analysis of physiologic mechanisms by use of tracer quantities of radioactive material has been made possible and has come into extensive use. Metabolic studies of the rare elements, as well as of calcium and phosphorus, have been facilitated, and information not otherwise obtainable has appeared in the literature. Understanding of the biologic applications of radioactivity depends upon the advances made in atomic physics.

1. Atomic Nucleus

NUCLEAR STRUCTURE

The nucleus of the atom represents all of the properties that distinguish the individual atom and by far the greater part of its mass.

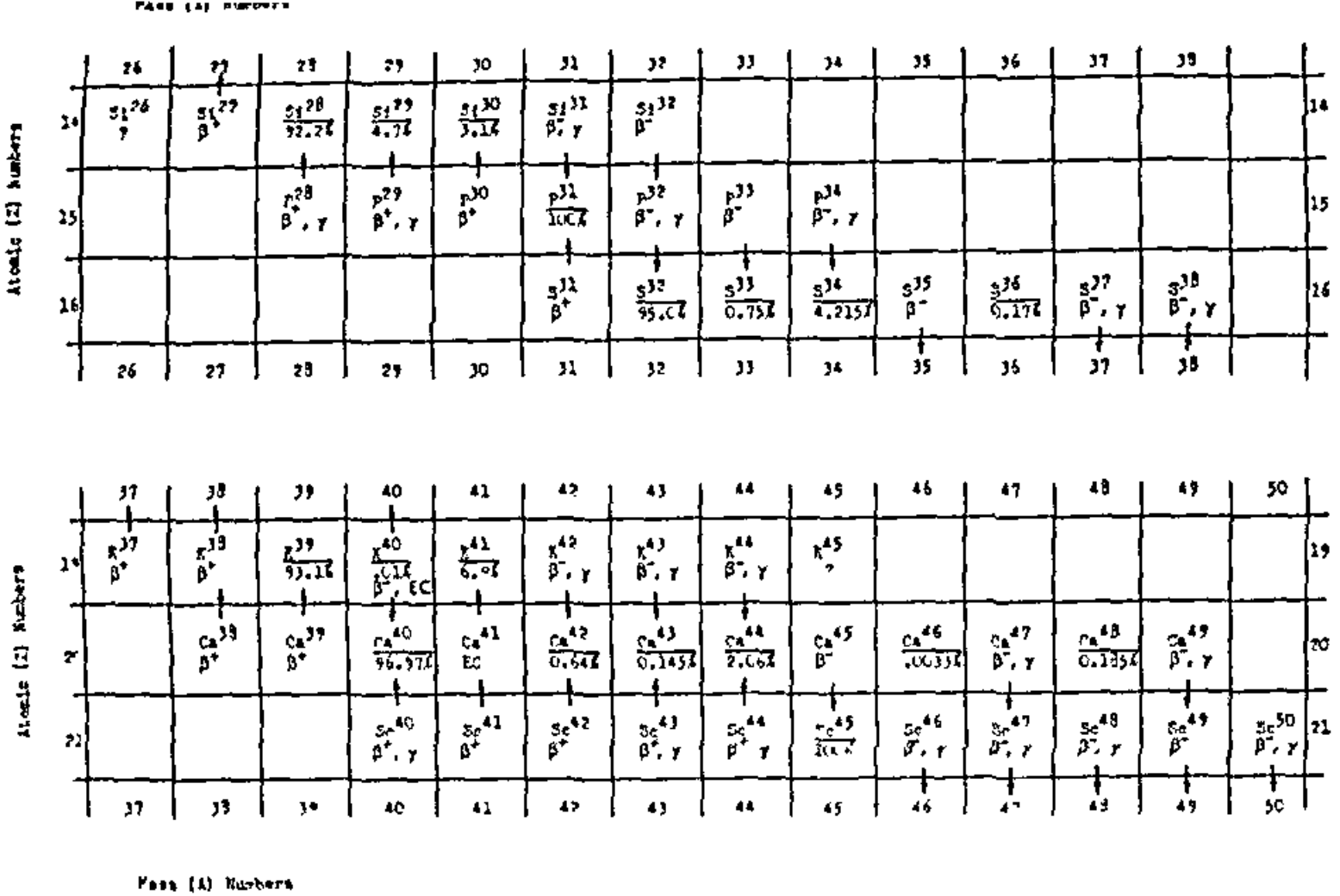


FIG 26 Partial chart of nuclides, to show isotopes of *phosphorus and of* calcium, with their relations to isotopes of neighboring elements Mass (A) numbers are in vertical columns, atomic (Z) numbers are in horizontal rows Shown are atomic numbers of isotopes, relative abundance in nature (in per cent), *and* type of radiation emitted Stable isotopes *are* underlined Arrows indicate pathways and direction of spontaneous decay with nature of decay (α, β^-, and electron capture, EC) Alpha decay does not occur in the elements shown (Data derived *from* Table of Isotopes, Strominger, *Hollander, and* Seaborg, Rev Mod Physics, 30 585)

Around it revolve electrons, common to all atoms, each carrying the negative electronic charge *e*. It is not within the scope of this volume to enter into the details of nuclear structure, but certain features are essential to orientation in the subject matter of this chapter.

The nucleus contains two kinds of elementary particles, *protons* and *neutrons;* either serves as the unit of mass; to each is assigned *mass number* 1. The important difference between them is that each proton carries a positive charge, equal in magnitude to the negative electronic charge *e*, while the neutron carries no charge. Each element in the periodic table has a different number of *protons* in its nucleus; this is its *Z* or *atomic number*, which characterizes the element.

The *neutron*, also a constituent particle of the nucleus, adds to mass in proportion to the number present and thus contributes to the nuclear mass, but not to its charge. The sum of the number of protons in a nucleus, plus the number of neutrons, is the *A* or *mass number;* this together with the atomic number or nuclear charge characterizes the *nuclear species*.

An *isotope*, or *nuclide*, is a single nuclear species of a particular element. A given element may have as few as three or as many as fifteen or more isotopes; all have the same atomic number, contributed to by the protons constant for each element, but different mass numbers, determined by the sum of protons and neutrons. An isotope, or nuclide, may be *stable* or *radioactive*.

RADIOACTIVITY

Radioactivity depends upon the emission of energy in the form of *radiation;* this results from a condition of instability in the nucleus, which seeks a level of stability. Emission of energy from a given nucleus is in one or more of three forms: (1) *alpha particles;* (2) *beta particles*, or electrons, either negatively or positively charged; and (3) *gamma rays*, or electromagnetic radiation. Alpha or beta radiation is associated with *decay* of the element, resulting in spontaneous *transmutation* to another element. The *physical half-life* of a radioelement is the time in which the amount of a

radioisotope is reduced by decay to half its initial value. Gamma radiation, while originating from isotopes undergoing alpha or beta decay, is not itself a part of decay or transmutation, since it represents neither loss of mass nor of charge.

Alpha particles carry two positive charges and are composed of two protons and two neutrons; they are identical with the nucleus of a helium atom. Their emission is the result of *alpha decay*, which occurs predominantly in elements with atomic numbers greater than 82. Emission of an alpha particle from the nucleus of an atom results in the loss of two positive charges and a reduction of 4 in mass number; the net effect of this is *transmutation* to a different element. Most of the heavy elements, with atomic numbers greater than 82, are unstable with respect to alpha decay.

Beta particles are identical with electrons and may be either positively (β^+, positron) or negatively (β^-, negatron) charged. For a beta particle to be emitted from a nucleus, which has none in its structure, an electron is created in the act of its emission. This requires that either a neutron or a proton, commonly the former, must undergo transformation to the other; a neutron thus gains a positive charge, while releasing a negatively charged electron, and becomes a proton; a proton loses its positive charge, releases a positively charged electron, and becomes a neutron. At the same time, in either case, there is released another particle, of infinitely small rest mass, and without charge—the *neutrino*. When *beta decay* of a nucleus occurs, the mass number remains the same, while the atomic number is either increased (β^- decay) or reduced (β^+ decay) by one. Beta decay thus results in transmutation to another element; formation of another isotope of the same element is not a possible consequence of beta decay.

Gamma rays represent electromagnetic radiation emitted from the nucleus of the atom, as *photons*. Except for differences in wave length and, consequently, in penetrating power, gamma rays are identical with light rays; they have properties similar to those of X rays. The emission of gamma rays from the nucleus occurs as a concomitant of an excited state of the nucleus, ordinarily associated with alpha or beta decay, or both; it represents an adjustment of

the energy relations within the nucleus, and its terminal state is de-excitation or return to the ground state. Gamma radiation, per se, leads to no change in the mass or the charge of the nucleus and, thus, to no transmutation of elements.

There are two situations in which only gamma rays are emitted from an atom; one of these has bearing on the ordinary use of isotopes. This is the phenomenon of *electron capture* (EC), by which is meant that the nucleus captures an electron from the outer shell of the atom; this results in the transformation of one proton to a neutron, and the reduction of the atomic number by one. An excited state of the nucleus may result, accompanied by the emission of gamma rays. Of the isotopes in common use (Table 5), those that undergo transformation to other elements, with emission of gamma rays only, are: Be^{7} to Li^{7}; Sr^{85} to Rb^{85}; and Ba^{133} to Ce^{133}. In addition, the transition of F^{18} to O^{18} and of Na^{22} to Ne^{22} occurs by a combination of β^{+} decay and electron capture. The second phenomenon associated with gamma radiation alone is that of *isomeric transition* (IT), which represents a delay in adjusting to the ground state. Since the half-lives of isomeric states are generally short, this phenomenon is not important in the biologic applications of radioisotopes.

RADIOACTIVE ISOTOPES

The term *isotope*, or its synonym, *nuclide*, refers to any one of the forms an element may assume; a nuclide is characterized by the atomic number of the element and the mass number of the particular nuclide. The term isotope does not itself imply radioactivity; an isotope may be either stable or radioactive. A radioactive isotope may be designated as a *radioisotope* or *radionuclide*.

Many isotopes occur naturally; others are produced artificially; the number of known isotopes of all the elements is of the order of 1,000. New elements, as well as new isotopes, have been formed in the laboratory. In addition to the transmutations that occur naturally, as in the decay of the heavy elements, such as radium, transmutations may be induced. Of these events the most dramatic is nuclear fission, brought about in the atomic bomb, and resulting in radioisotopes of the 34 elements in the periodic system from zinc

(element 30) to the rare-earth europium (element 63). In some instances several isotopes of the same element may be formed as fission products. Other radioisotopes are produced by bombardment of the nucleus with particles, more commonly neutrons, which add to the mass of the nucleus that absorbs them, resulting in a new isotope of the same element, with an increase by one in the mass number. Other examples of transmutation are *deuteron-induced*, *alpha particle–induced*, or *proton-induced*. *Neutron bombardment* requires a source of *neutron flux*, commonly a *nuclear reactor*. Induction by deuterons, alpha particles, or protons requires acceleration of the particles, in order that they may acquire sufficient energy to enter the nucleus under bombardment. A variety of high-energy accelerators is now available for research in particle physics, as well as for the formation of new isotopes. An interesting development is that of an electron linear accelerator which produces very high energy electron beams; this can be used to produce an array of isotopes not otherwise available. An important source of radioisotopes is by extraction and purification of fission products present in the fuel waste from atomic reactors.

2. Radiation and Bone

From the wealth of material now available, we have chosen to limit ourselves to: (1) the metabolism of radioelements having a special affinity for the skeletal system; (2) the pathophysiologic effects upon bone of internal and of external radiation; and (3) the use of radioisotopes as tracers in the study of bone.

Fixation of Radioelements by Skeleton

Experiments with radioactive elements have firmly established the concept of the skeleton as a dynamic system. In addition to the changes in structure and in distribution of the bone mineral mediated by cellular activity, every ionic grouping in the mineral is subject to replacement. Moreover, many elements have a special affinity for the bone matrix and may remain fixed in it for long periods of time.

Replacement may occur at the surfaces and within the structure of crystals. It is appropriate to consider these phenomena as re-

sulting from two processes: rapid ion exchange in and on the crystal surfaces and slow incorporation of ions within the crystals by intracrystalline exchange. Both occur continuously; to them must be added the formation of new crystals during growth and reconstruction of bone; remodeling crystals by recrystallization is also believed to occur.

Because of the manner in which bone is renewed by erosion and deposition, there is a definite pattern to which exchange conforms. In the area of growth and remodeling, where vascularity is greatest and all the crystals are of recent origin, the equilibrium between the intercellular fluids and the mineral phase is rapid and nearly complete; in such areas the uptake of administered radioisotopes is at its maximum. In the older osteons of adult compact bone, and in lamellar bone, the crystals are partially isolated and less able to incorporate new ions, either physiologic or foreign, into the bone mineral. Even in such bone, however, there is diffuse deposition of radioelements, by *long-term exchange.*

The most striking sequel of administration of radioelements with an affinity for bone is their deposition in new and incompletely mineralized osteons. The result is a spotty distribution of discrete foci of intense concentration; these are the areas frequently given the designation of *hotspots.*

The concept of intracrystalline exchange has important bearing on the behavior of elements associated with the bone mineral. These elements, studied largely by the use of radioisotopes, may be divided into two groups: (1) those elements or ionic groupings which can enter into a surface reaction with the bone crystals but, because of space considerations or electric charge, cannot be incorporated within the structure; and (2) those which can substitute for the normal constituents of bone in the interior of the crystals.

An example of the first category is uranium. This element can be held at the surface as the water-soluble uranyl ion, UO_2^{++}. Since this remains exposed to the body fluids, it is rapidly removed and excreted; it has a relatively short effective half-life. Complex ions, such as citrate, are also fixed at the surfaces; it is still uncertain whether carbon dioxide, as $CO_3^{=}$, is held on the surface, or

in the crystals, or both. Substitutions of ions, both in the surfaces and in the interior of crystals, is taken advantage of in experimental work and in clinical investigation by administration of the radioisotopes of calcium and of strontium, chiefly Ca^{45}, Ca^{47}, and Sr^{85}. Radium and lead are also capable of such substitution.

In addition to the elements associated with the mineral of bone, a considerable number, of which the prototype is plutonium, do not react with, or substitute in, the bone salt, but do exhibit a predilection for deposition in the bone matrix. Except for S^{35}, which substitutes in the sulfated mucopolysaccharides in the ground substance of bone and cartilage, the nature of the combinations by

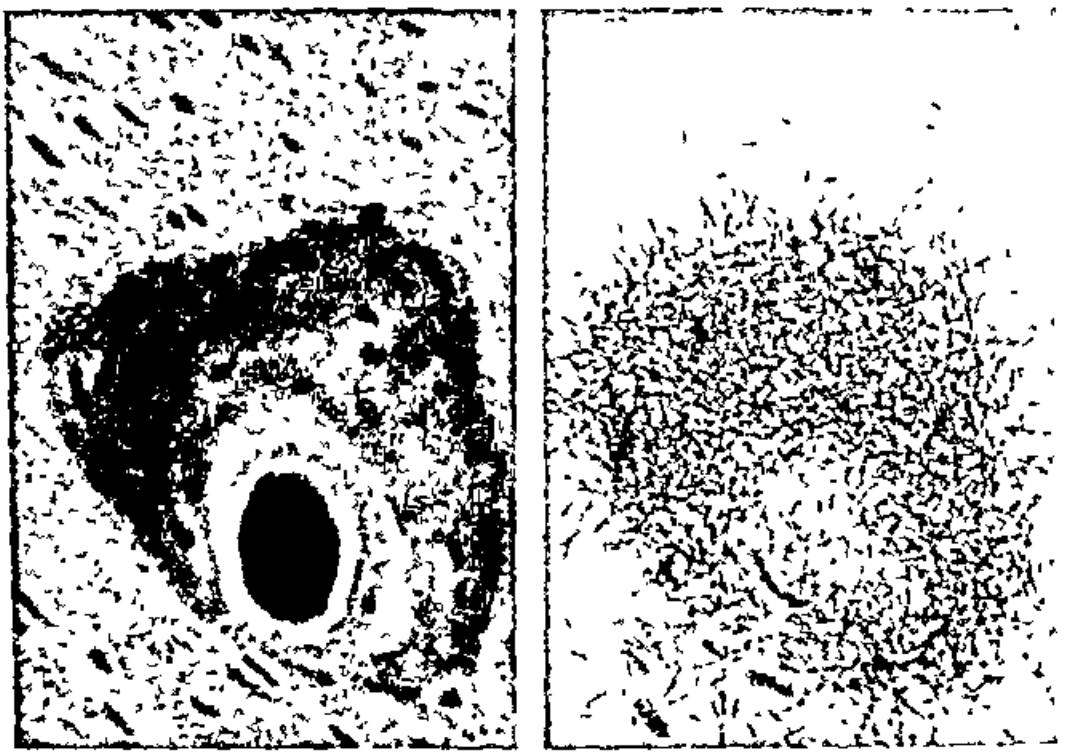


FIG 27.—Microradiograph (*left*) and alpha-track autoradiograph (*right*) of single osteon from compact bone of a woman who received 41 weekly injections of *radium chloride at age 32 and died 24 years later. Bone embedded in methyl* methacrylate and sectioned at 100 μ, with high-speed rotary saw, without further treatment. Microradiograph made with 10 KV X rays on Eastman Kodak 649–0 spectroscopic plate. The osteon is labeled throughout, owing to length of period over which radium was administered. The hypercalcification around the haversian canal in the microradiograph is characteristic of radium poisoning. ×236. (From original of Fig 2, Rowland and Marshall, Radiation Res., 11:304. Reproduced by courtesy of the publishers.)

TABLE 5

RADIOISOTOPES OF SPECIAL INTEREST IN RELATION TO THE SKELETON

Z No.	Element	Isotope	Half-life	*Radiation* Type and Energy, Mev	*Decay Type and* Transition Product	Interest for Bone
1	Hydrogen (tritium)	H^3	12 3 y	β^- 0 018	β^- to stable helium-3	H^3 thymidine to label nuclei
4	Beryllium	Be^7	53 6 d	γ 0 479	EC to stable lithium-7	Fixed on crystal surfaces
6	Carbon	C^{14}	5568 y	β^- 0 155	β^- to stable nitrogen-14	Fixed as $CO_3^=$
9	Fluorine	F^{18}	112 m	β^+ 0 6	β^+ and EC to stable oxygen-18	Substitutes for OH^-
11	Sodium	Na^{22}	2 6 y	β^+ 0 54 γ 1 28	β^+ (89%) and EC (11%) to stable neon-22	Diffuse deposition
	Sodium	Na^{24}	15 0 h	β^- 1 39 γ 1 38 2 76	β^- to stable magnesium-24	Diffuse deposition
12	Magnesium	Mg^{29}	21 h	β^- 0 42 γ 1 35	β^- to 2 3 min radioaluminum-28	Fixed on crystal surfaces
15	Phosphorus	P^{32}	14 3 d	β^- 1 71	β^- to stable sulfur-32	Exchanges for P^{31} as $PO_4^=$
16	Sulfur	S^{35}	87 1 d	β^- 0 167	β^- to stable chlorine 35	As $SO_4^=$ in ground substance
20	Calcium	Ca^{45}	164 d	β^- 0 255	β^- to stable scandium 45	Exchanges for Ca^{40}; β^- emitter
	Calcium	Ca^{47}	4.7 d	β^- 1 94 0 66 γ 1 31	β^- to 3 4 day radioscandium-47	Exchanges for Ca^{40}; γ emitter
38	Strontium	Sr^{85}	64 d	γ 0 513	EC to stable rubidium 85	Exchanges for Ca^{40}; γ emitter
	Strontium	Sr^{89}	51 d	β^- 1 5	β^- to stable yttrium 89	Exchanges for Ca^{40}; β^- emitter
	Strontium	Sr^{90}	27 7 y	β^- 0 54	β^- to radioyttrium-90	Fallout hazard
39	Yttrium	Y^{90}	64 h	β^- 2 26	β^- to stable zirconium-90	Fixed in areas of resorption
56	Barium	Ba^{133}	7 2 y	γ 0 36	EC to stable cesium-133	Exchanges for Ca^{40} on crystal surfaces

Half-lives m, minutes h, hours d, days, y, years

Radiation types α, alpha particles, β^-, beta particles (negatrons), β^+, beta particles (positrons), γ, gamma radiation, Mev, million electron volts

Decay, β^-, β^+, α, EC (electron capture)

TABLE 5—*Continued*

Z No.	Element	Isotope	Half-life	Radiation Type and Energy, Mev	Decay Type and Transition Product	Interest for Bone
57	Lanthanum	La^{140}	40 h	β^- 1.32 1.67 2.2 γ 1.6 0.81 0.49 0.33	β^- to stable cerium-140	Fixed by bone matrix
88	*Radium*	Ra^{226}	1620 y	α 4.78 γ 0.188	α to radioradon-222 (94%) γ *in 6% of transitions*	Exchanges for Ca^{++}; induces neoplasms
89	*Actinium*	Ac^{227}	21.6 y	α 4.92 β^- 0.046	α to radiofrancium-223 (1.2%) β^- to radiothorium-227 (99%)	Fixed by bone matrix
90	Thorium	Th^{234}	24.1 d	β^- 0.192 0.104 γ 0.090	β^- to 6.7 hr protactinium-234 (UX_2)	Fixed on crystal surfaces
92	Uranium	U^{233}	162,000 y	α 4.80	α to radiothorium-229	Fixed as uranyl ion, UO_2^{++}
94	Plutonium	Pu^{239}	24,400 y	α 5.147 5.1 γ 0.035 0.050	α to radiouranium-235	Fixed at endosteal surfaces; *found in osteoclasts*

which elements may remain fixed for extended periods in the *organic matrix is poorly understood.*

Table 5 summarizes information concerning the radioactive isotopes of elements of special interest in relation to the skeleton. Those included have assumed importance, either because they constitute a hazard to man, or because they are useful as tracer elements in the study of bone; others, known to be bone-seekers, but of theoretical interest only, are omitted. The table includes the half-lives of the elements listed; the types of radiation emitted, with their energies; the types of decay and transition products; and some information concerning the interest in relation to bone. The *half-life* of a radioisotope is the length of time for it to reduce its radioactivity, by decay, by one-half.

FISSION PRODUCTS

Nuclear fission has assumed importance, not only because of its destructive properties, but also because of the liberation of radioactive fission products in the testing of nuclear weapons, and because of the much greater hazard from the possible use of these weapons, in quantity, in war. In the detonation of a bomb, cleavage of a uranium or a plutonium nucleus leads to the formation of two atomic nuclei, each of which is unstable and radioactive. The mixture of fission products emits both beta particles and penetrating gamma rays. It includes a number of elements whose half-lives vary from a fraction of a second to many years; the intensity of the radiation falls off sharply with time, owing to the rapid decay of the radioisotopes with short half-lives. Among the 34 fission-product elements, some of which are represented by more than one radioisotope, there are 14 whose rates of decay are expressed in terms of days or years, and which are produced in relatively large amounts by fission. Almost all the radioactivity remaining in a fission-product mixture after it has decayed for a week comes from the isotopes of these elements. Of these, some may be stored in bone for twenty-five to five hundred days or more, and thus constitute a potential hazard; only two are absorbed to a significant degree by the gastrointestinal tract. These are strontium and barium, both

members of the alkaline earth series; only strontium is found in sufficient amounts among the fission products to constitute a significant hazard; for this reason, and because of the amounts of strontium liberated into the biosphere by atom bomb tests, current attention is centered on the radioisotopes of this element. Moreover, of the radioisotopes of strontium liberated by nuclear fission, only Sr^{90} has a long half-life (27.7 years); that of Sr^{89} is fifty-one days. Thus of all the fission products, the long-term hazard to man by virtue of uptake and retention in bone, narrows down to the one radioisotope, Sr^{90}.

STRONTIUM-90

Most of the Sr^{90} found in man and animals, as a result of contamination of the atmosphere, gains entrance by ingestion in foods; the pathway is air to plants, via soil or direct uptake by leaves, plants to man, either direct or via the flesh or milk of animals ingesting contaminated plants. While the behavior of strontium in plants and in the animal body is similar to that of calcium, there are differences that are significant in the hazard to man of Sr^{90}. Comar and others have shown that differential behavior of calcium and strontium occurs mainly in (1) absorption from the gastrointestinal tract, (2) reabsorption in the kidney tubules, (3) transfer across the placenta, and (4) secretion into milk. In each case there is discrimination against strontium in favor of calcium, and Comar has introduced a *discrimination factor*, to apply to a particular physiologic process that brings about the differential behavior. In an approach to a mechanism for discrimination, Schachter and Rosen have found that while calcium and magnesium are transported *in vitro* from the mucosal surface of the gut by a mechanism limited in capacity and dependent upon oxidative phosphorylation, this is not operative for strontium. The strontium/calcium ratio in bone is approximately equal to that in the blood. It appears that bone, *in vivo*, does not discriminate against strontium. The strontium/calcium *observed ratio*, diet to bone, has, however, been found to be low in all animals studied, owing to discrimination in intestinal net absorption and in urinary excretion. The kidneys discriminate against strontium by a preferential reabsorption of cal-

cium; the net result is that the organism has some protection against retention of Sr^{90} in the body.

An alternate route of entry is through the lungs. Inhalation of aerosols of fission products or of plutonium will lead to much the same distribution in the body as if the substances had been absorbed by the gastrointestinal tract. Aerosols of insoluble materials are however, poorly absorbed through the lungs, being largely moved out of the bronchial tree by ciliary action and swallowed with the sputum. Only about one-twentieth of the present amounts of Sr^{90} in the skeletons of the adult population, resulting from *fall-out* from the biosphere, can be attributed to inhalation. Other routes of entry, of lesser importance, are by contamination of wounds and by percutaneous absorption.

REMOVAL OF ISOTOPES FROM BONE

Many efforts have been made to find methods by which radioactive elements, once absorbed into the body, can be prevented from deposition in bone, or having been so deposited, can be removed from the bone and from the organism. Two types of approach are under current consideration: (1) the use of carrier agents, such as zirconium, which form colloidal aggregates with the radioelement in the blood, then to be removed from the circulation by excretion through the kidney; and (2) complexing and chelating agents which form soluble, non-ionic, readily excreted complexes with the radioisotope. As to carrier therapy, zirconium has been used successfully to remove plutonium, yttrium, and cerium; it is most effective if given at a time when the blood level is highest, i.e., as soon as possible after it enters the blood. It has not been useful in increasing elimination of strontium or of thorium.

The chelating agents, especially polyamino acid compounds, *such as ethylenediamine tetraacetic acid (EDTA) and diethylene*triamine pentaacetic acid (DPTA), have proved very useful for removing a number of radioisotopes from the body, including plutonium, thorium, yttrium, and the rare earths, such as cerium. Again, these agents have not been effective against strontium or radium.

In no case is it to be expected that removal of an element from the stable bone mineral will be practicable, since this would require virtual demineralization of the bone. The above methods may be expected to reach only the isotopes still in the blood, or at the most, those in the readily exchangeable portions of the bone mineral.

3. Effects of Radiation upon Bone

Units of Radioactivity and of Radiation

The unit of radioactivity is the *curie*, abbreviated c. This is approximately the activity of 1 gm. of radium, and the unit affords a measure of the radioactivity of any nuclide in comparison with that of radium. For small amounts of radioactivity the terms millicurie, mc, and microcurie, μc, are used.

The effect of radiation in biologic systems is determined by the energy transferred to the system. The two units in common use for expressing this energy are (1) the *roentgen*, abbreviated r, and (2) the *rad*. Another unit, used with respect to calculations involved in radiation protection, is the *rem*, originally an abbreviation for roentgen equivalent, man; still another unit is the roentgen equivalent physical, abbreviated *rep*. The roentgen unit is used for X or gamma radiation only and is based on the ionization produced in air; the rad is used for all types of radiation, internal or external, and refers to amounts of energy absorbed. The rad is the more generally useful term; it is defined as 100 ergs absorbed per gram of tissue. The roentgen is the exposure dose; the rad is the absorbed dose.

External Radiation

The bones of adults have a high degree of resistance to radiation. The bones of children, however, and particularly the growth apparatus of the long bones, are susceptible to injury. Such injury is not a hazard of ordinary diagnostic roentgenography, but overexposures may result from therapeutic radiation; uninformed use of fluoroscopic equipment may also be responsible for harmful effects.

In experimental animals, total body irradiation will usually result in death from doses below those necessary to cause damage to bones. Much higher doses, restricted to local areas, may, however,

cause extensive changes in the bones. The parallel columns of cartilage cells in the epiphyseal cartilage plate become disarranged; the cartilage cells swell; and the matrix takes on a mottled and fibrillar appearance. Growth ceases, mainly as a result of interference with the ingrowth of blood vessels and with the replacement of the hypertrophic cartilage. Osteoblasts may disappear, assuming the characteristics of reticular cells. If the damage to the growth apparatus is slight, recovery will occur within a few weeks; larger doses may result in permanent impairment of growth. The changes to be expected in growing children are similar to those found in young animals.

Damage to the bones of adults is most often seen following very heavy and localized X-ray treatment or similar radiation from other external sources. Spontaneous fractures of the ribs, of the neck of the femur, and of the jaw have been reported under such circumstances; the lesion responsible for the fractures is necrosis. Serious marrow changes are produced at doses much lower than those approaching the threshold for damage to bone substance; damage may be done to the vascular channels, both of bone and of bone marrow, at somewhat higher doses than those that affect the hemopoietic function and the formed elements of the marrow.

INTERNAL RADIATION

The effects of radiation from radioactive elements deposited within the skeleton are essentially similar to those of external radiation. Alpha and beta particles and gamma radiation result in similar effects; observed differences depend more upon the distribution of the element in bone and the intensity of radiation than upon differences in the type of radiation. Alpha particles, which have a very short range, do not penetrate as far as do beta particles. Gamma radiation influences tissues at much greater distances from the source. On the basis of an equal amount of absorption per unit volume of tissue, alpha particles are considerably more effective than is beta or gamma radiation. For this reason, alpha emitters, such as plutonium, deposited in the endosteal surfaces of bone, in close proximity to the bone marrow, are especially destructive to the marrow as well as to the bone.

BIOLOGIC EFFECTIVENESS

The biologic effectiveness of internal radiation, besides being dependent upon its quality and intensity, is determined by a combination of the *physical half-life* of the radioelement and its *biologic half-life*, which is the time required for the biologic system to eliminate one-half of the substance that has been introduced into it. The *effective half-life* of a radioisotope in a biologic system is the resultant of a combination of its physical and its biologic half-life. For those elements fixed in bone, the effective half-life in this location may be assumed to be relatively long; in the case of the long-lived isotopes it may far exceed the length of life of the individual.

The *relative biologic effectiveness* (RBE) refers to a comparison between different types of radiation and different elements or isotopes. The concept has a limited usefulness, since the biologic effectiveness of any radiation depends on many factors in addition to the characteristics of the source of radiation, including biologic half-life, distribution and retention in tissues, and differences in susceptibility of tissues. Moreover, the RBE of alpha to beta radiation changes markedly with the level of dose. As a useful guide, the relative biologic effectiveness of the various types of ionizing radiations, other conditions being equal, may be approximated as follows: X rays, gamma rays, beta rays, and electrons, 1; alpha particles, 10; for tissues within the short range of alpha radiation the biologic effectiveness is some ten times as great as for other types of internal radiation.

RADIUM

There is now a great deal of information concerning the effects of radium deposited in the bones of man. Of that which remains in the bones over periods of years it may be assumed that all is eventually incorporated within the crystals of the bone mineral, substituting therein for calcium. Since radium remains within the bone mineral, mainly within the lamellae of compact bone at some distance from the marrow cavity, and since radium and its radioactive decay products emit chiefly short-range alpha particles, the effects on the bone marrow may not be prominent. The organic matrix of bone is relatively resistant to radiation; but over a period of years

considerable damage may be done. In severe cases of poisoning in radium-dial painters this has frequently been manifested early as necrosis of the mandible, maxillae, and temporal bones. Pathologic fractures in other bones have also occurred.

RADIATION AND BONE TUMORS

A manifestation of delayed tissue damage produced by internally deposited radioisotopes, including radium, is malignancy; the induction period for appearance of the tumors following uptake of radium has varied as much as from twelve to thirty years. Of the malignant tumors of bone, the most frequent has been osteogenic sarcoma.

Information is also accumulating concerning the body burden of radium in individuals who develop bone tumors after the long latent period following exposure. So far, no case has been reported in which less than 0.4 μc of radium, plus an undetermined amount of mesothorium, was left in the body of an individual developing an osteogenic tumor, who had been exposed to radium in adult life. On the other hand, one patient with a low terminal body burden of 0.8 μc developed a bone neoplasm. Radium had been given medically twenty-four years previously, and the total accumulated dose from mesothorium was estimated at 50 rads, with 1,100 rads from radium; these are dosage levels at which major skeletal damage in other patients has been observed.

The appearance of a bone tumor following radiation from an external source was reported in 1922; since then, many isolated cases have appeared. In a recent report, seventeen patients presented bone sarcoma, sixteen of these following irradiation for benign conditions. In another group of cases osteosarcomas were observed in patients given radiation for reasons other than benign conditions of bone.

The effects of radioelements other than radium on bone have been rarely observed in man; reliance must be placed on experiments on animals. Comprehensive long-range studies on the toxicity of plutonium (Pu^{239}), radium (Ra^{226}), mesothorium (Ra^{228}), radiothorium (Th^{228}), and strontium (Sr^{90}) are under way at the Radiobiology Laboratory at the University of Utah. Osteogenic

sarcomas are produced in a colony of inbred beagles following single injections of the isotopes. Radium and strontium are primarily deposited in areas where bone is being formed at the time of the injection, with some diffuse distribution; plutonium, thorium, and mesothorium localize on endosteal bone surfaces. Osteogenic sarcomas, dose-dependent and time-dependent, have been *found as a consequence of all these radioelements; they are frequent*ly multiple. The *relative biologic effectiveness* of the different isotopes depends mainly on the retained dose level of each.

Strontium, like radium, is incorporated within the crystals of the bone mineral, following an initial stage of rapid uptake by exchange for calcium ions at the surface. The incidence of bone *tumors, in rats and rabbits, is approximately proportional to the* dose of the radioelement. Single and multiple tumors and extensive metastases have been observed. Radioactive yttrium and cerium also produce sarcomas in the skeletal system, especially in the long bones. Following sublethal doses of phosphorus-32 a considerable proportion of the survivors develop malignant tumors, usually *osteogenic sarcomas.*

The pattern of bone tumor response, as observed following introduction of Sr^{89} or Sr^{90} in CF_1 mice is as follows: (1) There is a latent period, following which bone neoplasms, mostly osteogenic sarcomas, develop rapidly, and in many cases as multiple neoplasms. The latent period is not shortened by increasing the dose. (2) *There is no linear relation between dose and response.* (3) *The* data are consistent with the existence of a threshold below which sarcomas are not induced.

4. Radioactive Tracers in Study of Bone

The use of radioactive elements as tracers in the study of bone dates from the observations of Chiewitz and Hevesy, in 1935, when they demonstrated that there was a rapid uptake of phosphorus-32 by bone by exchange between the phosphate ions of the bone and those of the blood. Since then, the use of tracer elements has been greatly extended and has become a common procedure in many laboratories.

There are two main categories in the use of tracer elements.

Either the element itself, as an ion or in an ionic grouping, may be administered; following this, its behavior in the organism, usually with reference to a specific system, may be observed. The second category is the use of a radioactive element combined in an organic compound which itself may have specific physiologic activity, such as that of a vitamin or a hormone; the distribution and metabolism of the compound are followed by observing the tracer elements. A variant of this is the administration of a labeled organic compound which has a specific metabolic pattern, or which may act as a building block in the synthesis of a more complex molecule; by this means complex reactions, and even cellular behavior, may be studied. Owing to the avidity of the skeleton for numerous elements, which has led to their being called bone-seekers, radioactive tracer elements have been much exploited; on the other hand, only a few labeled organic compounds have been used in studies of the metabolism of bone.

AUTORADIOGRAPHY

The principles of autoradiography are applicable to study of the distribution of radioelements in the organism in a variety of ways. The object containing radioactive material is placed in contact with a photographic emulsion, and, on development, an image is produced which provides visualization of the location of the radioactivity in the sample. On a gross scale, a whole bone may be cut with a saw, and the cut surface placed in contact with film; thus the distribution of the radioactivity may be viewed in either sagittal or cross sections of the bone. When desirable, serial sections may be made; in any case, studies of the deposition of radioelements in bone do not ordinarily permit decalcification.

Autoradiographs made from thin ground sections of cortical bone are easiest to interpret. The information can be expanded by histologic staining to disclose the location of the endosteum and cells, by microradiography to show the density of the mineral deposits, and by ultraviolet microscopy to reveal administered fluorescing substances incorporated in the new lamellae—all in a single section (see Frontispiece).

For study at the cellular level, high-resolution autoradiography

is required, and the photographic image on the film may be enlarged to any desired size. A method in common use is to apply the photographic emulsion directly to a histologic section; by this means, the section stained by conventional methods and the photographic image of the radioactivity may be viewed together under the microscope; a variation of this method is the use of a special emulsion that records the tracks of alpha particles from heavy elements deposited in the material. Special emulsions are now available for recording tracks of beta particles.

Autoradiography may also be used in conjunction with paper chromatography. In this case, the autoradiogram is made from the chromatogram, and the two methods together serve to identify the material responsible for the radioactivity.

For the most part, the information obtained by autoradiography is of a qualitative nature. Optical densities of autoradiograms have, however, been measured with a densitometer. On a microscopic scale, and with the use of high-resolution techniques, quantitative information may be obtained by counting alpha or beta tracks, or, for diffuse distribution of activity, by counting the number of darkened grains in the emulsion.

RADIOASSAY

Quantitative methods for estimation of the radioactivity in samples of biologic material require: (1) preparation of uniform samples; (2) counting of the number of disintegrations, per unit of time; and (3) expression of data, with appropriate corrections. The details of radioassay are highly specialized, and are dependent upon variables that are beyond the scope of this chapter. The end-result of radioassay is usually best expressed in terms of *specific activity*, i.e., the amount of radioactive element per unit weight of the element present, the weight to include both active and stable isotopes. In the fraction used to express specific activity the denominator is the unit mass of the element, which may be in terms of weight or of mols or millimols. The numerator may be expressed as percentage of dose; in arriving at this figure a correction is made, if necessary, for any decay that may have occurred between administration of the isotope and final count of the sample.

EXTERNAL COUNTING IN VIVO

When it is desired to determine the distribution of radioactivity in the skeleton *in vivo*, an external counting method may be used, by which observations are made at the body surfaces. For this purpose there is required a radioisotope which has a special affinity for the skeleton, and emits penetrating gamma rays. If the observations are to be made on living man, it is also necessary that the amounts required to be administered shall be without danger to the individual; this means that preference is given to a short-lived isotope.

The isotopes that meet these specifications, to a greater or lesser degree, and that have been used for clinical observations in man are Ca^{47} and Sr^{85}. Ca^{47} has been available only in limited quantities; it has proved useful in detecting lesions in the skeleton, at times before X-ray evidence is conclusive. Sr^{85}, which emits only gamma rays, is more readily available, and insofar as the skeleton is concerned, furnishes information comparable to that obtained by the use of an isotope of calcium. For studies of distribution in man, with limited applicability to the skeleton, Na^{24} has been used. It emits gamma rays, demonstrable by external counting.

ISOTOPES AND KINETICS OF MINERAL METABOLISM

The radioisotopes with a special affinity for bone have proved useful in the study of the kinetics of its mineral constituents. Modern applications to calcium metabolism are described above (chap. xi). By estimating the rate of disappearance from the blood, following intravenous injection of a radioisotope, and by estimations on bone, urine, and feces, it is possible to calculate the rates of accretion and of resorption, as well as the amount of the isotope in the exchangeable fraction of the bone mineral. These calculations are aided by the use of an electronic analog computer. In addition to its applicability to calcium metabolism, the method has also been used with strontium and with phosphate.

ISOTOPES AND ION TRANSFER

When Ca^{45} or P^{32} is administered intravenously to an animal, it is demonstrable, by autoradiography, within a few minutes and

in specific locations. These are chiefly the new and incompletely calcified osteons incident to the remodeling of compact bone; rapid deposition also occurs subperiosteally and subendosteally in the growing bones of young animals. This occurs by *ion transfer*, a term applied to the movement of ions without specifying the mechanism. When this occurs as a one-for-one exchange, without a net change in the solid phase, it is called *ion exchange*, which may be isoionic or heteroionic. There is no essential difference between ion transfer and ion exchange in bone; both occur passively in an approach to equilibrium with the fluids with which the crystal surfaces are in contact.

Although transfer of radioisotopes has been demonstrated autoradiographically only in the direction of blood to bone, it is commonly assumed that transfer occurs in both directions, and that the areas in which deposition is demonstrable represent the labile, reactive, or exchangeable pool of calcium and phosphate in the bones. If this assumption is correct, it is these areas that are responsible for the rapid transfer of calcium from bone to intercellular fluid and blood in the homeostasis of the calcium levels in the fluids of the body; a slower transfer of ions to blood occurs as a result of osteoclastic resorption of stable bone, under regulation by *parathyroid activity*.

In addition to the transfer of ions from the blood to areas where radioactivity is concentrated in the bones, a number of radioisotopes are deposited diffusely in association with the bone mineral; this has been called the *diffuse component* of radioisotope distribution, in contrast to the component concentrated in the new osteons and represented by the *hotspots*. The activity in the diffuse distribution of radium in bone samples from individuals who have carried radium twenty years or more has been found to be relatively uniform in cortical bone. The average magnitude of this activity is about one-half of the total body burden divided by the weight of the bones. The specific activities in the hotspots are, on the average, ninety times as great as in the diffuse component; the total amount carried in the hotspots is, however, approximately equal to that in the diffuse component, when the entire skeleton is taken

into account. Similar distributions are found for Ca^{45}; sodium, however, as Na^{22} or Na^{24}, is demonstrable only as diffusely deposited in short-term experiments; Na^{22} has been found concentrated in new osteons as late as four months after it was administered.

LABELED COMPOUNDS AND BONE

The distribution and fate of hormones and vitamins in the body has been, to a limited extent, studied by means of labeled compounds. Examples with reference to the skeleton are *estrone-16-C^{14}*, *tritiated estradiol* (H^3-6, 7-estradiol), *C^{14}-ergocalciferol*, and *tritiated amino acids*.

Evidence that endosteum is a target for estrogens, characteristic for the mouse, is based on the localization of radioactivity in the skeleton when other tissues have been cleared of this activity. The patterns of distribution and metabolism of radioactive estrone are different from those of tritiated estradiol; the relation of the hormone to osteoblastic activity has not been established. When C^{14}-labeled vitamin D was administered to rachitic rats, tissues intimately connected with the turnover of phosphate, such as bones, intestines, and kidneys, contained significant amounts of the labeled vitamin.

Tritiated thymidine is rapidly coming into use as a label for cell nuclei. Thymidine enters the structure of deoxynucleic acid (DNA) during interphase. When tritiated thymidine is administered, the nuclei of cells undergoing division are labeled, and they may, subsequently, be identified by microautoradiography. This procedure is currently being employed in the study of the life-cycles and transformations of the cells of bone, including pre-osteoblasts, osteoblasts, osteocytes, and osteoclasts.

The deposition of matrix material has been studied by autoradiography after injection of various precursors, such as *C^{14}-bicarbonate*, C^{14}-glucose, C^{14}-proline, S^{35}-methionine, C^{14}-methionine, H^3-leucine, H^3-methionine, and H^3-glycine. Of these, H^3-glycine has proved to be a useful tracer of newly-formed matrix. In sites of bone growth in adult mice, the glycine label has been found to give a strong reaction in the cytoplasm of osteoblasts within thirty

minutes after injection of the tritiated amino acid, when the preosseous tissue reacts only moderately. At four hours the radioactivity in the osteoblasts has diminished, while the preosseous tissue is heavily labeled. From these observations it has been concluded that osteoblasts elaborate a matrix precursor within their cytoplasm and release it to preosseous tissue. Direct evidence for a collagen precursor, tropocollagen, has been obtained by addition of C^{14}-proline to a suspension of osteoblasts; this was followed almost immediately by the appearance of protein-bound C^{14}-hydroxyproline, although collagen fibers were not detectable until a day later.

CHAPTER XIII

Postfetal Osteogenesis

Osteogenesis continues as the normal growth of bone through adolescence; it takes part in the physiologic turnover of bone throughout life. It becomes highly active in the repair of fractures and of other injuries to the skeleton. It occurs normally as extraskeletal ossification in certain locations; as a pathologic process it is observed in the ectopic formation of bone. It can be brought about experimentally or in the treatment of fractures by transplantation of cells with osteogenetic potencies or tissues capable of leading to formation of new bone by *induction;* bone formation may also be observed in tissue and organ cultures.

1. Tissue and Organ Culture

The earlier work on tissue culture of bone deals chiefly with the embryology of the skeleton. A review of the literature up to 1937 by Bloom describes the techniques used to study the behavior of cells *in vitro.* Culture techniques have been extended to include tissue culture proper, cell culture, and organ culture. Of these, tissue culture is the only method, thus far developed, applicable to the study of postfetal osteogenesis. In a successful culture the cells appear to revert to an undifferentiated state; they retain their osteogenetic potencies.

Osteogenesis from Bone Anlagen

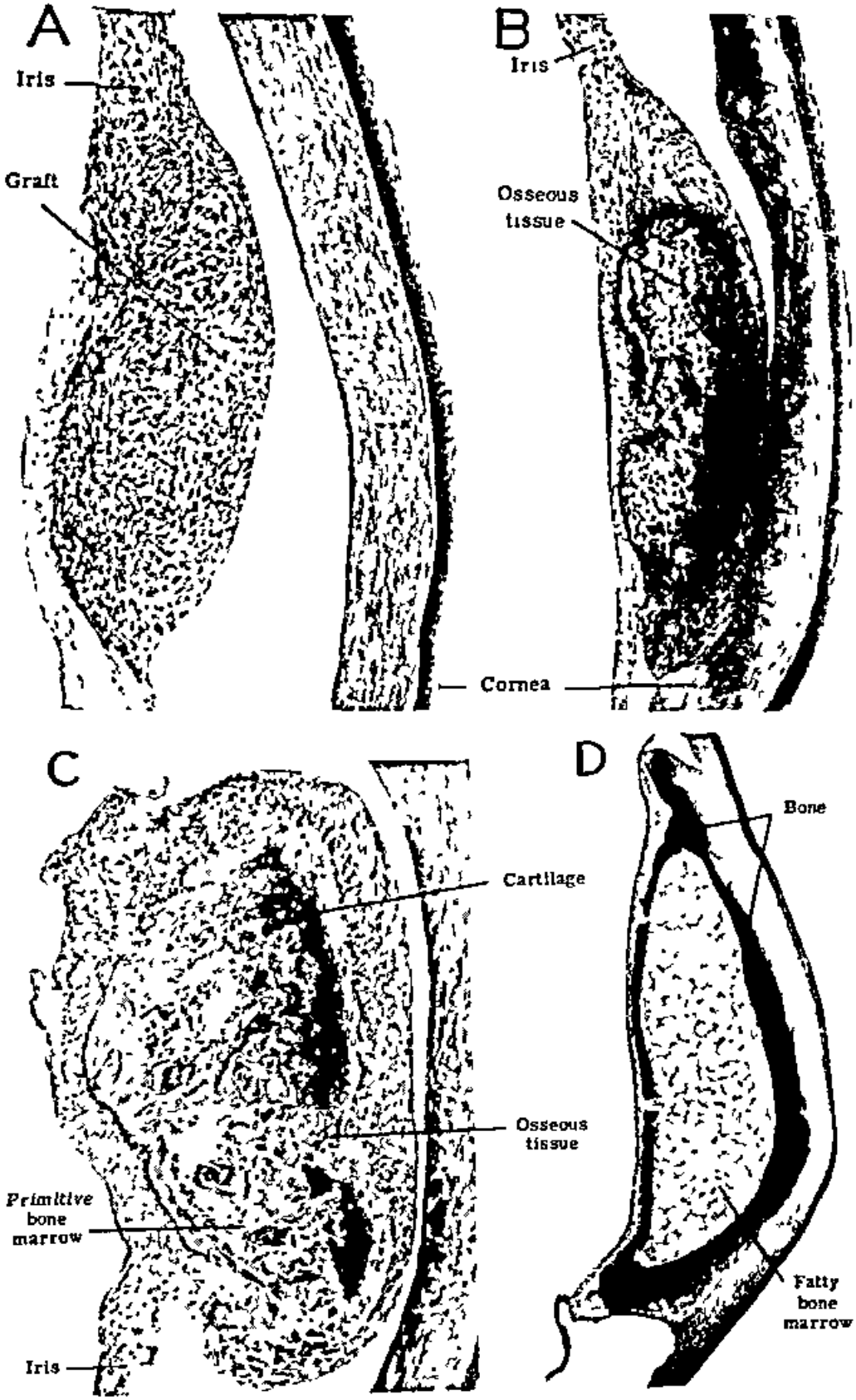
The earliest rudiment of the skeleton is an axial condensation of the mesenchymal connective tissue in the core of the limb bud. With organ-culture methods, fragments of this tissue as small as thirty cells in volume, when cultivated, will produce the cartilage model of a bone. The cells first become enlarged, then spread apart by a homogeneous intercellular matrix, and finally are organized

as bone. The shape of a bone such as the femur is predetermined in certain cells of the limb bud as early as the third day of life of the chick embryo. The cartilage model is absorbed and replaced by bone through the formation of an intermediate tissue described as *chondro-osteoid*. Under these conditions cartilage is incompletely calcified and transforms directly into bone tissue. If a chondrogenetic core of the limb bud of an early chick embryo is disintegrated by enzymatic digestion of the intercellular matrix, thereby reducing isolated organs to suspensions of single cells, the suspended cells reaggregate and differentiate into masses of cartilage. Clusters formed from mixed suspensions of chondrogenetic and myogenetic cells produce an inner mass of cartilage and an outer sheath of muscle tissue. From this it has been concluded that the discrete limb bud of the early chick embryo is capable of re-establishing a tissue-like association and of resuming its characteristic histotypic development.

Fell demonstrated that tissue-culture and organ-culture methods permit study of the response of isolated tissues and organs to vitamins. There is a direct effect of vitamin A on skeletal tissue, specifically on the intercellular matrix of the cartilage. When a culture medium contains an excess of vitamin A, differentiation of early chick-embryo limb buds is interfered with by arrest of growth. The cellular zones of cartilage differentiate, and periosteal bone develops, but the cartilage becomes soft and gelatinous and loses its characteristic basophilic and metachromatic staining reactions. In late fetal mouse limb bones, the cartilage matrix quickly disappears, leaving free chondroblasts during the rapid resorption of bone. The surrounding soft tissue grows profusely, but all that remains of the bone is a sheet of amoeboid cells containing a few fragments of bone and cartilage. Vitamin A acts only on viable cells; cartilage models heated to 45° C., just sufficient to prevent growth, lose all the effects of the vitamin.

OSTEOGENESIS FROM OUTGROWTHS OF BONE TISSUE

A fragment of bone tissue about 2 mm. square, obtained from nine- to twelve-day infant rats, will produce an outgrowth in tissue



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culture by the coverslip method within two days. The outgrowth, representing both bone and bone marrow, consists of an inner ring of spindle-shaped cells surrounded by an outer ring of free amoeboid cells, such as macrophages, eosinophil myelocytes, and heterophil myelocytes. The original explant can be excised at four days and reimplanted in other nutrient media, to obtain new generations of outgrowing cells. Each outgrowth can be either subcultured or transferred as a homogenous transplant to a host site, to determine its cell potencies for differentiation into bone cells.

After the cells of the outgrowth are separated from the explant of bone tissue and transplanted, a new ring of closely packed fibroblast-like cells appears in the center of the subculture. These cells are offspring of primitive mesenchyme, endothelium, reticular cells of bone marrow, or endosteum. Samples of bone and bone marrow, containing all these cells, produce better outgrowths and healthier cultures than do those scraped free of bone marrow.

Masses of cartilage appear by the third day; spindle-shaped cells are in the loose meshwork, where there is no bone or cartilage formation. By the sixth day the masses of bone, cartilage, or chondro-osteoid are circumscribed by a perichondrium-like layer of spindle-shaped cells. When outgrowths are transplanted to the anterior chamber of the rat's eye, cellular differentiation follows the same sequence as in a subculture, with the exception that there is less chondro-osteoid. Instead, the cartilage matrix is calcified, immediately invaded by blood vessels and perivascular connective tissue

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FIG. 28.—Development of bone in the anterior chamber of the eye of the rat, from transplants of outgrowth of tissue cultures of bone from a 12-day rat. Fragments of bone were cultured in rat plasma and embryo extract. The outgrowths were removed after 4 days *in vitro* and transplanted to the iris of normal rats. These outgrowths contained no bone or cartilage. *A*, after 1½ days in the eye, there was no cartilage or bone. *B*, after 2½ days in the eye, chondro-osseous tissue developed from the culture. *C*, after 6 days in the eye, much bone and cartilage and some marrow appeared. *D*, after 4 months, a "bone" with central fatty marrow developed. *A*, *B*, *C*, Zenker-formol, hematoxylin-eosin-azure II; ×70. *D*, formalin; silver nitrate-hematoxylin-eosin; ×28 (courtesy of J. H. Heinen). (From original photomicrographs of Fig. 120, Maximow and Bloom, Textbook of histology [6th ed.; Philadelphia: W. B. Saunders Co., 1952]. Reproduced by courtesy of the publisher.)

cells, and finally replaced by new bone within two weeks. The sequence is that of endochondral ossification. The process is organotypic; the cells of the transplanted outgrowth are capable of developing and becoming organized into an ossicle, filling the anterior chamber of the eye (Fig. 28).

The osteogenetic potency of the outgrowths is lost if separated from bone tissue for more than fourteen days before transfer to the anterior chamber of the eye. Nearly all samples will produce bone after eight days of cultivation, but after this time the cells revert to fibroblast-like forms and produce dense masses of fibrous connective tissue instead of bone.

CALCIFICATION OF NEW BONE IN VITRO

Chondro-osteoid and osteoid tissues appear in cultures of bone anlagen and bone outgrowths, chiefly because the amounts of calcium and phosphorus are insufficient to promote continuous deposition of bone salt. Transplants of outgrowth in the eyes of rachitic rats do not exhibit calcification in either cartilage or bone. The same tissue can be made to calcify within a few days if the rachitic host is treated with parenteral injections of a solution of sodium phosphate or with vitamin D.

FACTORS INFLUENCING OSTEOGENESIS IN ORGAN CULTURE

Remodeling of bone and development of the internal architecture are guided by intrinsic forces present in limb buds as well as by external forces. Cartilage differentiates, *in vitro*, from small fragments of a limb bud by an inherited capacity of the cells for self-differentiation without the necessity for an external force. Cultures of perichondrium subjected to pressure can be stimulated to produce an excess of cartilage. Organ culture has proved a useful method for study for intrinsic and extrinsic factors in skeletal development; explants of avian sternum will form a keel in the absence of pectoral musculature, for which it normally serves as an attachment; a knee joint appears in the cartilage model of the leg from which the muscles have been removed; an explant of mouse sternal buds with rib stumps differentiates into a normal segmental

structure, whereas when the rib stumps are removed before explanting, the sternum ossifies as a continuous double bar without segmentation. For the present it is known that extrinsic factors play an important role in osteogenesis; both biochemical and mechanical influences are present. Fracture healing occurs in tissue culture just as in the normal skeleton.

Sodium fluoride and other substances that inhibit enzymes seem to have no effect on osteogenesis in tissue culture. Alizarin Red S added to culture media for supravital staining permits fibrous connective tissue growth and chondrogenesis but inhibits osteogenesis. Hyaluronidase causes stunting of growth of cartilage models and diminished basophilia of the cartilage matrix, without any specific effect on osteogenesis. The distribution of phosphatase in hypertrophic cartilage and osteoblasts is the same in tissue culture as in the normal skeleton.

2. Transplants of Bone

Progress in tissue transplantation has made it increasingly clear that several biologic principles, to some degree interrelated, are essential to the successful transfer of bone tissue, as judged by the survival of a *bone graft*, or by its replacement by new bone formed by the host, with the end result, in either case, of anatomic and functional repair of a skeletal defect. Information to this effect has come from both experimental and clinical observations, the results of which may be put in a perspective of interest both to the biologist and to the surgeon.

The major principles to be taken into account are: (1) *osteogenetic potency*, or the ability to form bone, expressed as *osteogenetic activity* of bone-forming cells; (2) survival and proliferation of transplanted tissue or cells, giving rise to the formation of new bone originating from the transplant; (3) the *immune response* of the host, to the transplanted tissue or cells, which may be the major factor in determining whether the transplant will survive; (4) *induction* of new bone formation, under the influence of specific properties residing in the implant, conferring osteogenetic potency on the tissues of the host; and (5) a non-specific affinity of the host

for the interstices of an implant of devitalized bone or of an inert material, leading to its removal and replacement by bone.

The work of Medawar and of Burnet, for which the Nobel prize in medicine was awarded in 1960, has firmly established the thesis that the immunologic defenses of the organism against foreign proteins are responsible for rejection of homogenous transplants of skin. This mechanism is doubtless operative with respect to homografts of bone, although the fate of transplants of homogenous bone is determined in part by factors not applicable to skin grafts, in which the success of the procedure depends entirely upon the survival of the donor cells; such survival may, in fact, lessen the probability of acceptance of a bone graft. For reconstructive surgery of the skeleton, when viability is not essential, and only an absorbable framework may be required, it is often possible to use implants of devitalized and preserved homogenous or heterogenous bone, or even of inert substitutes for bone.

OSTEOGENETIC POTENCY

A connective tissue having the capacity to form bone has *osteogenetic potency;* when this characteristic becomes manifest, the cell exhibits *osteogenetic activity.* Osteogenetic potency may be inferred and osteogenetic activity observed in many situations in the organism, most prominently in embryonic and postfetal formation and growth of bone and in the healing of fractures. We are here concerned with the ability of transplanted cells to survive, proliferate, and exhibit osteogenetic activity, and with the means by which osteogenetic potency may be conferred on cells, and osteogenetic activity elicited and observed.

IMMUNE RESPONSE

Of the factors entering into the problems of tissue transfers, that of the greatest interest, both experimental and clinical, is the immune response of the host to the implant; upon this depends the interpretation of many of the results obtained from experimental transfers of bone, as well as the feasibility of surgical transfers undertaken for the repair of defects in the skeleton. There is an increasing body of evidence that the general principles arrived at by transfer of soft tissues, including skin, are applicable also to bone.

membrane will survive if the pores of the membrane are small enough to prevent the passage of whole cells, but large enough to permit the diffusion of substances dissolved in the plasma, including humoral antibodies. There is evidence that the cells of living implants of heterogenous tissue are killed by circulating cytotoxins, produced by the host in response to the foreign proteins of the implant; the participation of humoral antibodies in the immune response to homogenous transplants is not excluded, although Curtiss *et al.* failed to detect circulating antibodies in rabbits injected with soluble or insoluble portions of homogenous bone.

The mechanism of the reaction of the host to homogenous transplants of bone is not clear. In the case of skin transplants, Medawar has characterized the delayed reactions as fundamentally cellular as opposed to humoral, and has stated that they depend upon "the activation, deployment, and peripheral engagement of the lymphoid cells." There is no doubt that homografts of solid tissues lead to inflammatory reactions, in which round cells and histiocytes predominate. Descriptions of the histologic features of the inflammatory reactions to homotransplants of bone in rabbits are given by Bonfiglio and his collaborators.

Enneking has reported histologic studies of the response of rats to autogenous and homogenous transplants of bone. Until the fifteenth day after transplantation there was no difference in the response of the host. After the fifteenth day only the proliferations of autogenous transplants consistently survived and aided in repair. The majority of homogenous transplants (34/52) invoked a major inflammatory host response, which obliterated the periosteal proliferations and prevented repair or replacement of the transplants. A smaller number (11/52) brought about a minor inflammatory response, which did not prevent the reactive bone of the host from crossing the transplant-host junction and repairing and replacing the transplant. A still smaller number (7/52) were accepted as though they were autogenous. The differences in the reactions of the host are probably attributable to the degree of inbreeding of the Sprague-Dawley rats used in the experiments. Enneking has also investigated the effect of whole body X-irradia-

sue treated in such a way as to destroy its antigenicity and then preserved in bone banks, in which case the other factors concerned in the transplantation of bone are of primary importance.

INDUCTION

Induction has been defined, and its role in the development and growth of bone has been discussed in chapter iii. Essentially, *induction* is the influence that one tissue may have upon another in close contact with it, as a result of which the second tissue is induced to exhibit activities not previously in evidence. An example, relevant to this chapter, is the formation of bone following implantation of bone from another individual. In such instances the implant does not itself proliferate and form new bone, but it may produce an effect upon the cells of the host to cause them to differentiate into osteoblasts and to organize in the form of a bone. The study of induction is aided by the use of biologic systems which are not complicated by an immune response on the part of the host. For this reason autogenous implants are favorable for observation of induction phenomena; the anterior chamber of the eye also provides a favorable environment, even for homogenous transplants, since interference by cell-borne antibodies is reduced.

Induction systems for bone formation generally obey laws governing cellular differentiation. The inductor is transmitted by a specific tissue, such as growing cartilage or urinary bladder epithelium, for a specific period of time. This may require a long exposure or latent period and may have a relatively short period of action. Normally the inductor is produced by proliferating cells, but it can emanate from resting, degenerating, or devitalized tissue. The inductor is believed to be transmissible over a short distance, rather than diffusible and extractable. The cells being induced to form bone are always connective tissue cells at a receptive stage of development; resting or adult cells can be induced to form bone rarely if at all. Holtzer has observed induction of cartilage by embryonic spinal cord in tissue culture, even after interposition of a millipore membrane; in the living animal whenever new bone is

produced by induction, this requires physical contact and the inductor is transmissible only between adjacent cells.

There are many theories concerning the mechanism of induction. We prefer to follow Weiss, who postulates progressive recruiting of cells for a given type of differentiation, spreading from a focal area like an infectious wave; cells which have attained a certain differentiated character can communicate their state to their neighbors, which then pass it on further, and so on down the line. This should require physical factors as well as chemical agents, participating in many sorts of combinations; there must be many degrees of specificity and complexity, employing a great variety of mechanisms.

CRITICAL CONDITIONS FOR TISSUE TRANSFERS

Many tissues, both of skeletal and of nonskeletal origin, have been tested for osteogenetic activity or for the ability to produce new bone by induction, by transfer of samples to isolated soft parts of the body or to defects in bone. The results are conditioned by: (1) the tissue transferred; (2) whether the donor tissue comes from embryonic, young, or adult animals; (3) whether it comes from a tissue in which latent potencies have been reactivated by injury; (4) whether the transplant is autogenous, homogenous, or heterogenous; (5) whether fresh or preserved tissue is used, and the state of the tissue when transferred; and (6) the nature and state of the host bed. The reaction produced by preserved tissues depends upon the method of preservation and the period of storage; whether the tissue had been simply frozen, or frozen at very low temperatures; whether the tissue had been coagulated by heating, boiling, or chemical solutions, or dehydrated before transplantation; and whether the donor tissue or its cells, if living, provoke an immune response from the host.

In mammals, skin is one of the few tissues that will survive, proliferate, and produce new cells after transplantation from one part of the body to another in the same individual during postfetal life; homografts of bone will vascularize, proliferate, and survive for a limited time only, owing to the immune response of the host. Cor-

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nea, fascia, cartilage, and undifferentiated connective tissue cells will survive autogenous transfer, and at times even homogenous transplantation; either they are non-antigenic, or inaccessible to cell-borne antibodies, or they resist their effects; acceptance of homogenous transfers must in some cases be related to the quantitative, as well as to the qualitative immune response on the part of the host. Homogenous transplantation of cancer cells, with vascularization, proliferation, and survival, is commonplace; the use of inbred strains of mice is chiefly responsible for the success of this procedure.

TRANSPLANTS OF OSTEOGENETIC TISSUE TO EXTRASKELETAL SOFT PARTS

Many kinds of host beds can be used to study osteogenesis under conditions in which there is no possibility of contact with preexisting osteoblasts. Transparent chambers may be placed in the rabbit's ear, or under the skin of the mouse, as receptacles for transplants. As in the case of the anterior chamber of the eye, the degree to which they will accept homogenous tissue may be related to their ability to exclude cell-borne antibodies. A favorite site for transplants of bone, or of tissues to be tested for induction of bone formation, is the subcapsular space of the kidney. Autogenous transfers to this site may survive and lead to results similar to those observed in the anterior chamber of the eye; homogenous transfers, unless from littermates or from inbred strains, more commonly lead to new bone formation by induction.

Transplants have also been made to muscle, brain, mesentery, testis, synovial membrane, and subcutaneous tissue; the success of such transplants depends upon the same factors as in the case of the subcapsular space of the kidney. The universal requirements of any transplant for the production of new bone are: (1) that the host tissues establish contact with the donor cells; (2) that the transplant leads to a proliferative response of new capillaries and undifferentiated connective tissue cells from the host bed, in addition to such proliferations as may be produced by the transplant itself; and (3) that it does not lead to an immune response, on the part of the host, capable of destroying the implant; this require-

ment is best met by autogenous transplants, more rarely by homogenous implants, and virtually never by heterogenous transfers except when devitalized and preserved.

Tissues that will not produce new bone following fresh autogenous transplantation are: fascia, ligaments, muscle, tendon, elastic cartilage of the external ear, and semilunar cartilage of the knee. The formation of bone follows autogenous transplants of periosteum; bone marrow, with or without cancellous bone; epiphyseal and articular cartilage; compact bone; and fibrocartilaginous callus after an injury to the bone of the donor.

The nature of the bone formed following an autogenous transplant differs with the tissue transferred to the new location. Periosteum from young growing animals includes an active osteoblastic layer and will produce new bone consistently after transfer to the anterior chamber of the eye. Periosteum from an adult animal will either produce no new bone at all or very little following transplantation. The same tissue removed several days after a fracture contains proliferating osteoblasts and has osteogenetic activity. A transplant of osteogenetically active periosteum to the anterior chamber of the eye produces a mass of compact bone, without an intermediate stage of cartilage and without the appearance of bone marrow. Such a reaction is histotypic; it forms only one kind of tissue.

Survival and proliferation of autogenous tissues, themselves capable of forming bone, may also be observed when bone marrow or cancellous bone, or a mixture of the two, is transplanted to the anterior chamber of the eye of the rat. Transplantation of the fibrocartilaginous callus after a fracture leads to a similar result. In either case a fibrocartilaginous mass results, to be replaced by bone and bone marrow; such a reaction is organotypic, since it forms a complete ossicle. The anterior chamber of the eye does not differentiate between autogenous and homogenous tissues, owing to the freedom of the aqueous humor from lymphocytes; transplants from other animals of the same species may be equally well accepted. Implants of the outgrowth from tissue cultures from bone of the same species also lead to the formation of bone in the an-

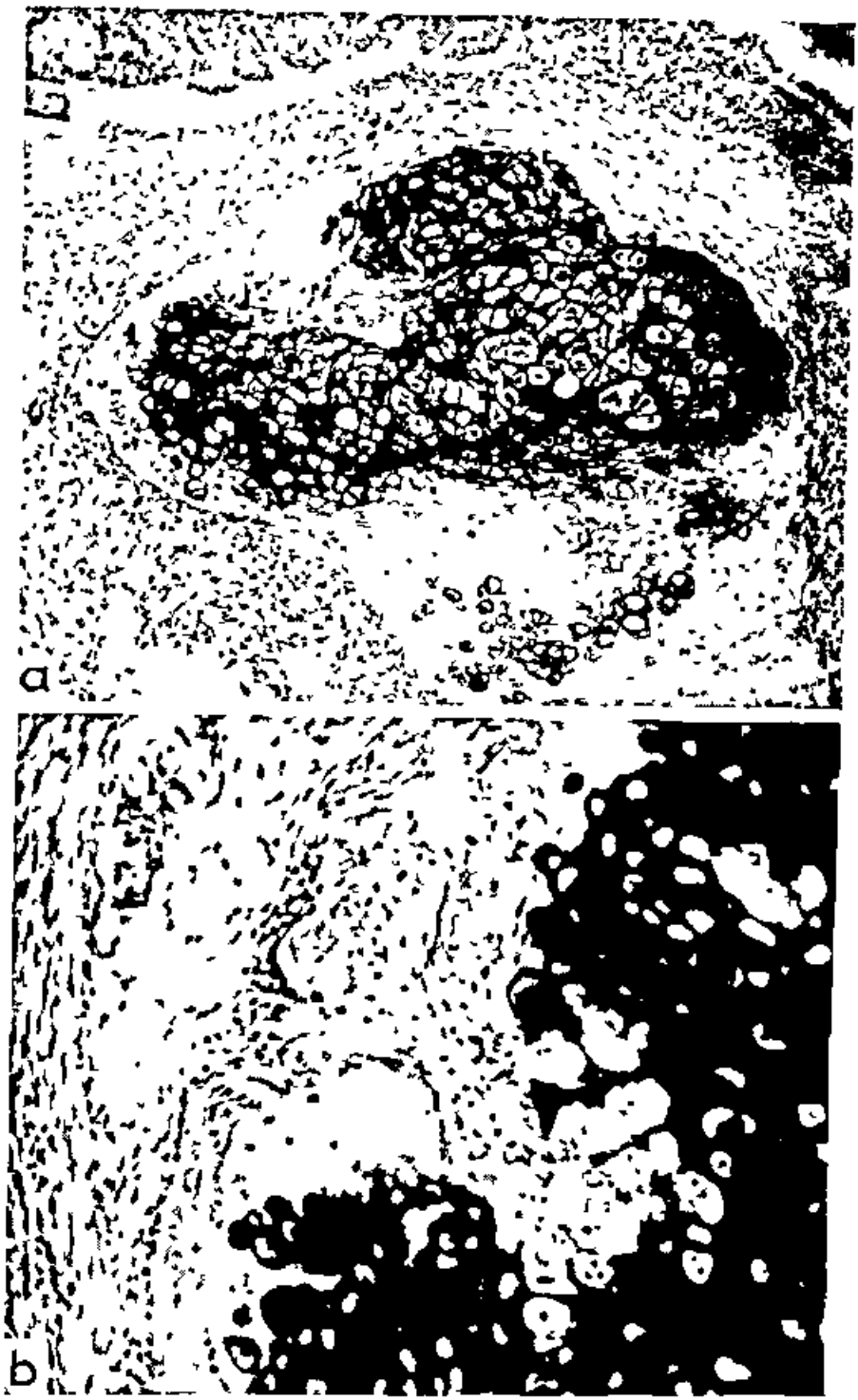
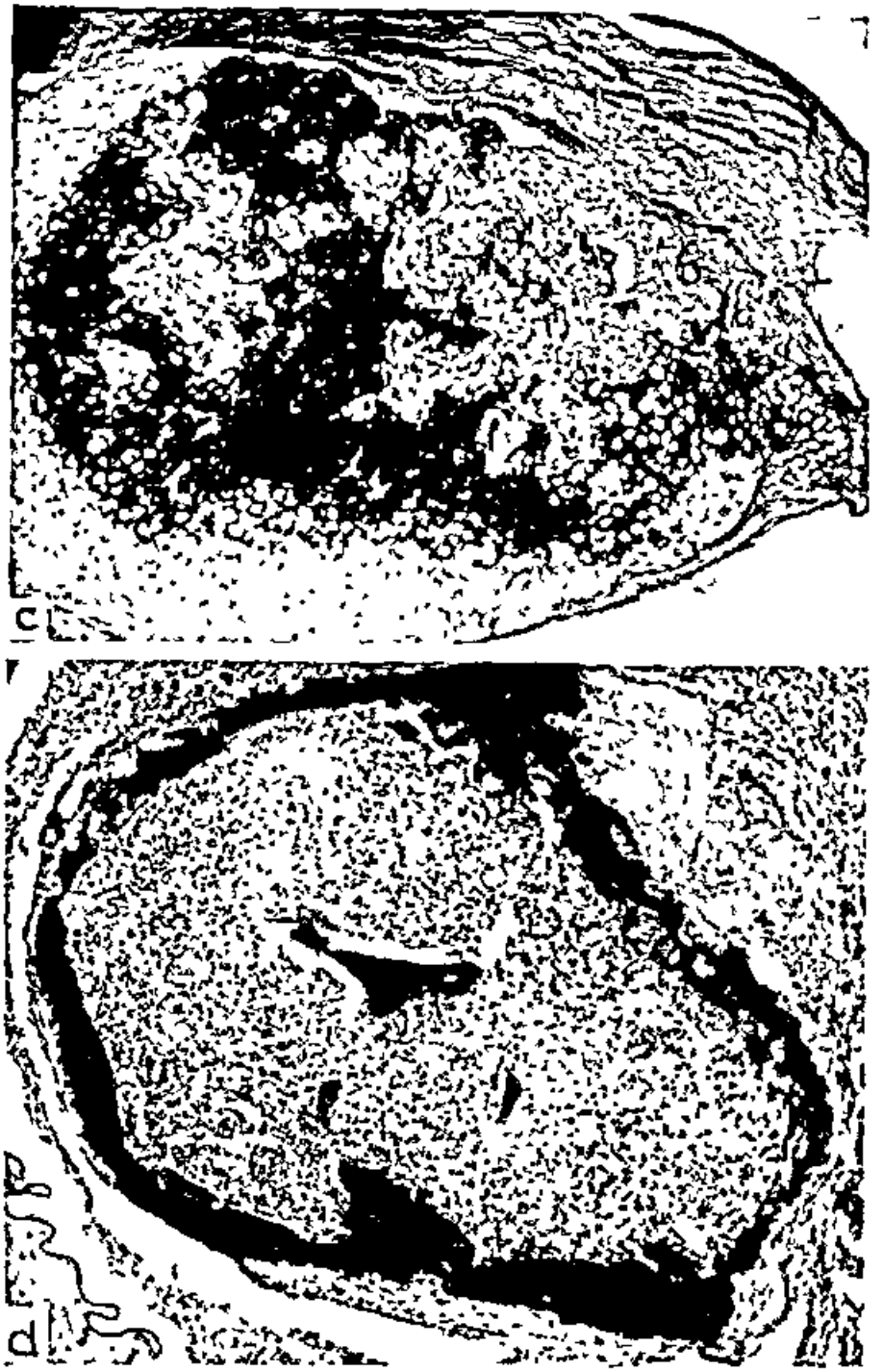


Fig 29.—Development of bone in the anterior chamber of the eye of the rat, from autogenous transplants of fibrocartilaginous callus. (*a*) Fragment of fibrocartilaginous callus encapsulated in granulation tissue after 7 days in the eye. At this stage, there is a sharp line of demarcation between the tissues of the donor and of the host The surface of the transplant shows metachromatic staining, but there is as yet no ingrowth of new vessels or absorption of the transplant. ×96. (*b*) Earliest invasion of the donor tissue after 11 days in the eye. The beginning new-



bone formation occurred with the first ingrowth of new blood vessels and in association with the absorption of the donor cells ×191. (c) Transplant after 17 days in the eye; further absorption of cartilage can be noted, as well as a thin wall of new-bone formation on the surface of the donor tissue. ×68 (d) End-product of the transplant after 27 days in the eye. Spongy bone has been reconstructed into a wall of compact bone. The result is a permanent complete ossicle filled with active bone marrow. ×71. (From original photographs of Fig 8, *A*, *B*, *C*, *E*, Urist and McLean, J. Bone & Joint Surg., 34A:453–55. Reproduced by courtesy of the publisher.)

terior chamber of the eye; again the reaction is organotypic, and a complete ossicle results (Fig. 29).

The behavior of transplants of epiphyseal cartilage is of particular interest. This tissue consists chiefly of hyaline cartilage, and is a remnant of the cartilage model of embryonic bone, engaged in endochondral growth. Following autogenous or homogenous transplantation to the eye of the rat it continues to produce new bone by endochondral ossification; new bone arises, however, not from transplanted cells, but from ingrowing cells of the host.

Transfers of autogenous compact bone to the eye exhibit little ability to form new bone; usually a new layer of bone is formed on the surface of the transplant, as a result of a combination of osteoblastic activity on the part of the transplant together with induction, which causes the connective tissue cells of the host to differentiate into osteoblasts and form new bone by apposition. Transfer of autogenous compact bone to any new location in the body leads to death of all or most of the osteocytes; survival and proliferation of osteoblasts, together with induction, account for the success of such transplants. Fresh homografts in higher mammals do not ordinarily survive, owing to the immune response of the host; early death of a homograft, with consequent reduction in its antigenic potency, is conducive to success of the graft; formation of bone under these circumstances is prima facie evidence of operation of the induction process, as it is also in the case of preserved and stored heterogenous bone.

TRANSPLANTS TO HOST BED OF BONE TISSUE

The most favorable conditions for survival of transplants of osteogenetic tissue are afforded by a fresh autogenous graft of cancellous bone, including bone marrow, to a well-prepared host bed of bone. Such transplants are commonly used in clinical surgery for *fusion of joints, internal fixation to produce union of fractures,* and filling of surgical defects. These conditions offer a high degree of probability that some of the transplanted tissue or of its cells will survive, proliferate, and produce bone; to this is added the probability that the transplant may cause the cells of the host to become

engaged in osteogenesis by induction. While conditions responsible for new bone formation by induction are unknown, except that both physical and chemical factors are involved, tissues other than fresh autogenous bone are considered less active and less desirable for reconstructive surgery. Experimental and clinical observations, however, leave no doubt that non-viable materials are effective irrespective of limited knowledge of how or why they accomplish their purpose. In Table 6 materials currently under investigation have been classified as bone implants, derivatives, and substitutes,

TABLE 6

MATERIALS USED FOR EXPERIMENTAL BONE SURGERY

TRANSPLANTS
- Fresh autogenous bone
- *Fresh isogenous bone (from identical twin)*
- Embryonic bone (for temporary survival)

IMPLANTS—Homogenous or heterogenous bone
- *Pretreatment*
  - Refrigerated in plasma
  - Frozen (*−30° C.*)
  - Freeze-dried
  - Boiled in physiologic salt solution
  - Autoclaved
  - Preserved in:
    - Aqueous glycol
    - Beta-propiolactone
    - Merthiolate
  - Irradiated by:
    - Cathode ray
    - Cobalt-60

DERIVATIVES—Homogenous or heterogenous bone
- Decalcified bone
  - Extracted with EDTA
- Anorganic bone
  - Extracted with ethylene diamine
- Os purum
  - Extracted with potassium hydroxide
- Collapatite
  - Extracted with urea, saline, and ether
- *Extracted with hydrogen peroxide*

SUBSTITUTES
- Metal
- Methyl methacrylate
- Polyurethane
- Calcium sulfate
- Hydroxyapatite

and distinguished from transplants, because they exhibit either very little or no viability and an unknown capacity for induction.

Recent experimental observations suggest that inert materials, with neither viability nor capacity for induction, can assume the appearance of a bone transplant. Various parts of the skeleton of young individuals are able to incorporate inert materials in an involucrum and thereby bridge large gaps and restore the continuity of a bone. The skeleton of a non-growing or an adult individual is not able to produce the same amount of periosteal new bone and generally requires viable autogenous bone to bridge a gap. When there is no gap and only internal fixation is required, implants of homogenous or heterogenous bone, derivatives of bone, *or bone substitutes, may often be used with good results.* The extent to which implants of fresh, frozen, or lyophilized bone, or pre-

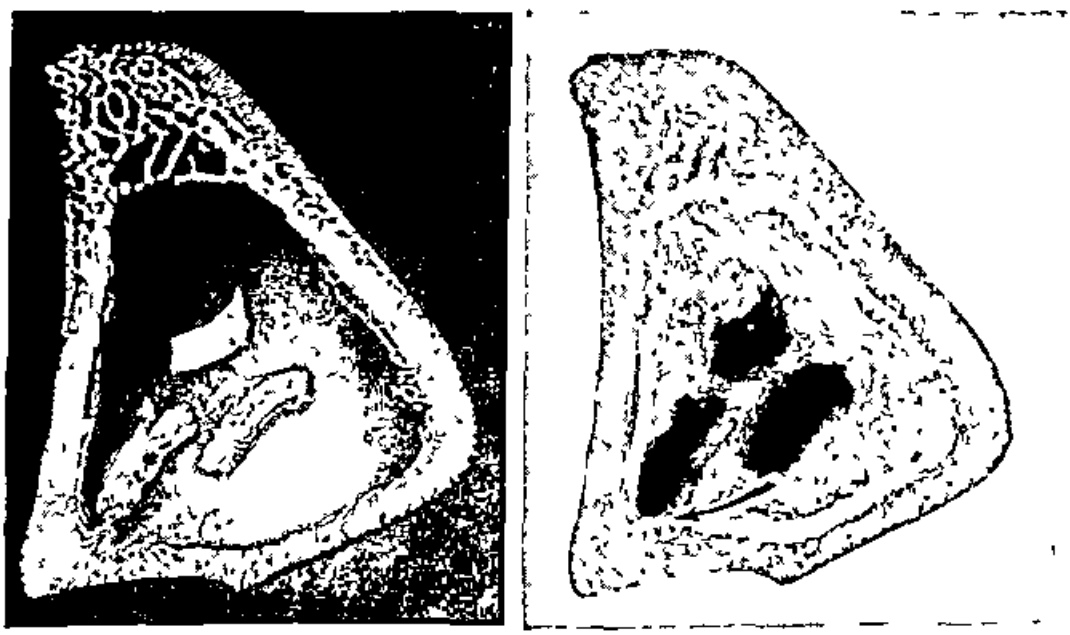


FIG 30.—Microradiograph (*left*) and autoradiograph (*right*) of a cross-section through tibia of a rabbit, with homogenous graft of bone inside the marrow cavity. The donor was a 6-week-old rabbit injected with 1 mc. of Sr^{90} 5 days before the *transplant into a host of the same age.* The tibia was excised 4 weeks later. The microautoradiograph, made with stripping film, records the radioactivity in the bone of the donor while the bone of the host shows only low level of diffuse radioactivity The arrow points to contact of the homograft with the bone of the host, where new bone is growing out from the endosteum. ×7. (From originals of Figs. 4A and 4C, Urist, MacDonald, and Jowsey, Ann. Surg., 147:137 and 138. Reproduced by courtesy of the publishers)

served bone can produce new bone by induction or may be superior to bone derivatives or substitutes, is often difficult to determine.

Radioisotope studies reveal little about induction but provide new information about the behavior of bone implants. Homogenous bone, either fresh or frozen, labeled with Ca^{45}, Sr^{90}–Y^{90}, or Y^{91} and implanted into bone defects in clean hosts, becomes joined to new bone produced by the host, through a cement layer or line. The radioisotopes of Ca and Sr label the mineral constituents of the donor tissue; they are also in solution in the tissue fluids of the host, but are not able to diffuse across the cement line. Correlated histologic sections, microradiograms and radiochemical analyses of the whole skeleton reveal that the donor tissue is reabsorbed in some places and covered with new bone in others; a large part of the radioisotope is excreted and a small part is redeposited systemically throughout the entire skeleton. There is no local transfer of bone mineral from the donor to the new bone of the host. The donor tissue accomplishes its purpose to enable the host to fill a defect, fuse a joint, or unite a fracture, when only a small fraction of its surface area has been resorbed. Whether the donor tissue is a transplant of fresh autogenous bone or an implant of homogenous or heterogenous frozen bone, by all known criteria it consists largely of dead tissue with empty lacunae. There is a definite affinity of the new bone of the host for interstices of an implant, whether it be transplanted dead or living bone, or a bone derivative or a substitute.

3. Extraskeletal Bone

When osteogenesis occurs in a part of the body where bone formation, in a particular species, does not normally occur, it is termed *ectopic* or *heterotopic* bone formation. Ectopic bone formation is an abnormality and is to be distinguished from *extraskeletal bone* in various locations in many animals, examples of which are leg tendons and fascia in the turkey, the laryngeal cartilages of man, and the penis of almost all mammals except the higher primates.

The number of organs and the many different kinds of pathologic lesions in which ectopic bone formation has been described seem not so important as that connective tissue elements, rather

than the specialized tissues, give rise to the osteoblasts associated with the appearance of bone. Nearly all the organs and tissues in the body, e.g., eye, brain, tongue, lung, heart, blood vessels, breast, testes, and anterior abdominal wall, are capable of harboring bone. Ectopic bone formation is very well known in connection with wound healing, *infectious processes, degenerative diseases, and* aging.

ECTOPIC OSSIFICATION IN MUSCLE

Ossification of muscle arises from disease or following injury. *Myositis ossificans traumatica* is a localized form developing rapidly in the deep muscles of the thigh or arm within a period of three weeks after a contusion and subsiding within six months. *Myositis ossificans progressiva* is a rare congenital or familial disorder, sometimes associated with microdactyly, continuing throughout the life of the individual, generally involving the muscles of the neck, back and hips. It is associated with inflammation, loss of striations of muscle cells, atrophy or reduction in the amount of cytoplasm, and proliferation of sarcolemmal sheath cells; later, after mitotic division, spindle-shaped connective tissue cells between the muscle fibers differentiate into new bone or cartilage or both. After six months, in nearly all cases, the bone deposit fuses with the cortex of the nearest bone. The new bone matures or becomes compact bone, and persists for years as an exostosis. Surgical excision frequently is followed by recurrence and involvement of a large segment of the muscle. The local conditions generating myositis ossificans are unknown.

Calcification of necrotic soft parts and muscle fibers closely adjacent to areas of osteogenesis has been regarded as evidence that calcification is a precursor of ossification. It is now clear, however, that ossification and calcification can be entirely separate and that calcium salts are not required for the initiation of osteogenesis, although calcified necrotic tissues are frequently areas in which ectopic ossification may ensue. Osteogenesis occurs in the healing of a mechanical injury or a pathologic lesion, but it always begins from newly proliferated connective tissue. Ectopic ossification is therefore not to be regarded as metaplasia of connective tissue elements

previously in existence in the lesion; not as a simple sequel to deposition of calcium salts in dead tissue; but as a reaction of perivascular connective tissue cells, growing into injured tissue from surrounding areas.

EXPERIMENTAL OSSIFICATION IN MUSCLE

Chemical injury to muscle tissue by injections of 40 per cent alcohol or acid alcohol in rabbits leads to the formation of fibrous scar, amorphous deposits of calcium salts, and in some instances, new cartilage or bone or both. Injections of 2.0 per cent calcium chloride produce calcification in bundles of collagen fibers, amorphous deposits of calcium salts, and calcified plaques of hyaline between the adventitia and the lamina elastica externa of the arteries outside the area of the damaged muscle. Some of these blood vessels exhibit lamellar bone formation associated with erosion of the calcium deposits. In the center of the lesion where there is liquefaction, caseation, necrosis, or disintegration of the structure of the tissue, the conditions are not favorable for bone formation. Osteogenesis appears where muscle cells atrophy, sarcolemmal sheath cells proliferate, and young fibrous connective tissue cells differentiate into chondroblasts and osteoblasts and form islands of new cartilage and bone.

Saline, alcoholic, and calcium chloride extracts of bone produce results not much different from those obtained with the extracting solution alone. When bone formation occurs the results may be attributed to physicochemical factors arising *in situ* rather than to an exogenous osteogenetic substance. It is common to find amorphous deposits of calcium salt, patches of hyalinized scar, and bizarre cells resembling preosseous, precartilaginous, or osteoid tissue. Unabsorbed injured muscle tissue, however, has the capacity to produce new bone formation by induction; Bridges and Pritchard were able to produce formation of cartilage, which eventually was replaced by bone, by implantation of small pieces of alcohol-devitalized smooth, cardiac, or skeletal muscle beneath the capsule of the kidney in rabbits. This is interpreted as induction of cartilage formation by the implant of dead muscle; it suggests that new bone

formation by autoinduction occurs when muscle is devitalized by injections of alcohol, with or without substances extracted from bone.

OSSIFICATION OF TENDON

Bone formation normally occurs in the substance of modified tendinous tissue at the point of bony insertions. Tendons also ossify following injury but such deposits are generally small and not as extensive as those developing spontaneously in birds. The process has recently been described in detail in the turkey. In these birds, during the first fifteen to twenty weeks of life, a new matrix, rich in acid mucopolysaccharide, accumulates between the collagen bundles of the tendons; the original tendon collagen bundles undergo changes in their chemical constitution; and osteons, similar to those in skeletal bone, appear as the result of formation of absorption cavities, which are then filled in with concentric lamellae of bone. All three of these tissues mineralize, and persist, with the result that the tendon eventually consists of mineralized collagen bundles, interspersed with calcified haversian bone.

ECTOPIC PERIARTICULAR OSSIFICATION

Patients with anterior poliomyelitis and other paralytic conditions frequently develop multiple foci of ectopic bone. This is usually preceded by extensive atrophy of bone, mobilization of large amounts of bone salt, excessive excretion of calcium and phosphorus in the urine, and formation of kidney stones. There is thus a sequence of events which seems to relate ectopic bone formation to the *atrophy of disuse* of skeletal bone; this sequence is not necessarily associated with any change in the levels of serum calcium, phosphorus, or phosphatase. The localization of formation of bone is determined by degeneration of muscles, tendons, and the soft-tissue elements of the joints; the result is a periarticular distribution of the deposits of new bone.

OSSIFICATION IN URINARY TRACT

In circumstances created by disease and from conditions following surgical operations, the urinary tract has a tendency to ectopic bone formation greater than that of any other tissue; this seems to

be related to the transitional epithelium of the calyces and pelvis of the kidney, of the ureter, and of the mucosa of the urinary bladder. Ossification occurs in the kidney of man within a fibrous capsule formed around old renal calculi. Deposits of bone can often be found after surgical incisions in the pelvis of the kidney or in the ureters. Bone has been produced experimentally by ligation of the renal artery in rabbits. It begins independently of any calcification and develops in the loose vascular connective tissue under the epithelium of the calyces.

When urinary bladder is transplanted to the fascia of the anterior abdominal wall the transitional epithelium proliferates and spreads in all directions to form a fluid-filled cyst. The roof of the cyst consists of the original implant and appears inert; the walls consist of spreading epithelium and induce formation of *lamellar bone all along the line of contact with the host bed. This is to be* regarded as an example of induction in which the bone arises from growing connective tissue cells of the host under the influence of the proliferating cells derived from urinary bladder. The nature of the inductor that is transmitted from one cell to another is unknown. It appears, however, that this induction system for bone is highly specific and dependent upon a factor transmitted from *dividing cells of transitional epithelium. Transplants of columnar* epithelium from other viscera proliferate and produce fluid-filled cysts but not new bone. The yield of new bone is high in the dog and cat, low in the guinea pig, and almost nil in the rat. The spleen is unfavorable as a host bed for osteogenesis for autogenous as well as homogenous transplants of urinary bladder. The fascia of the anterior abdominal wall and the capsule of the kidney are relatively favorable sites. With *homografts of urinary bladder in dogs a* reticulocyte-lymphocyte-plasma-cell response surrounds the spreading epithelium but never the new bone; this indicates that the bone cells are derived entirely from the body of the host.

CHAPTER XIV

Healing of Fractures

Mammals have inherited from lower vertebrates an extraordinary capacity to repair injury and replace missing parts of the skeleton. The proliferative reaction is equally vigorous in experimental and clinical healing of fractures. A bone is not simply patched together by scar tissue, as in the healing of most other organs. Bone repair is ordinarily so complete that it is impossible to find the area of a fracture or of a large defect one year after injury. New-bone formation is an automatic reaction to any form of injury to bone tissue. The injury and the reaction are so much a part of each other that the damaged tissue seems to introduce local factors which produce osteogenesis. The site of an otherwise unrecognizable bone injury can be located by the appearance of new-bone formation. The important unsolved problem of the physiology of fracture repair is the nature of the stimulus released by injury, and incitative to differentiation of connective tissue *cells into bone cells.*

The injured bone tissue immediately shows inflammation, then revascularization, and, finally, substitution by new bone that grows into it from adjacent endosteum and periosteum. In membrane bones the process is proliferation and direct extension of new bone from old bone. In long bones this involves preliminary formation of a model of fibrous connective tissue and cartilage through which osteogenesis is drawn into and across the fracture gap from each side. The fibrous connective tissue, cartilage, and bone are organized in the form of a complex structure termed *callus.* The interaction of cells and tissue resulting in healing of a fracture is an example of organization of diverse means to a common end.

NECROSIS OF BONE

In a large bone of adult man, with a closed non-comminuted fracture, at least 0.5 cm. of the shaft is damaged above and below the fracture line. If the fracture is comminuted and displaced, small and large bits of dead bone are found floating in the fibrinous clot. The necrotic bone is distinguished by pyknotic nuclei, autolyzing osteocytes, and empty lacunae. Increasing acidophilia distinguishes the devitalized matrix of bone from the undamaged tissue at the site of a fracture.

ORGANIZING HEMATOMA

As in wound healing in every other part of the body, fracture healing begins with the clotting of extravasated blood. The hemorrhages flow into the fracture gap and flood the soft parts, the bone marrow, periosteum, endosteum, and haversian canals. The organization of the blood clot begins within twenty-four hours on all surfaces, and fibrin is replaced by granulation tissue, in part of hematogenic origin, within a few days. Stinchfield and his associates observed that anticoagulants administered to rabbits at the time of a fracture caused delayed union. Both heparin and dicumarol have the same effect and prevent the formation of the fibrin clot between the bone ends. Adverse influences upon induction, cellular proliferation, and chondroitin sulfate synthesis are also possible, but the most important information that is gained from such experiments is that a trellis of fibrin is a mechanical necessity for ingrowth of cells capable of differentiating into fibrocartilaginous callus. It is by this means that a preliminary structure can form to bridge the gap whenever there is displacement of a fracture.

FIBROCARTILAGINOUS CALLUS

The torn ends of periosteum, endosteum, and bone marrow adjoining the fracture line supply cells of histogenic origin; these proliferate and differentiate into fibrous connective tissue, fibrocartilage, and hyaline cartilage. This growth of new tissue is contributed to also by cells of hematogenic origin—monocytes and possibly lymphocytes—that have responded to the stimulus of the inflammation and have participated in the organization of the

hematoma. At this stage, except for the predominance of cartilage, the healing closely resembles that of the repair of any tissue following an injury.

The appearance of cartilage in the callus is most prominent in fractures of long bones with displacement or large defects; here, the cartilage is a temporary filling material for later replacement and bridging of the defect by new bone. Small drill holes and thin saw cuts produce relatively little cartilage and are repaired chiefly by growth of connective tissue cells and osteoblasts from one surface to another. Although fibrocartilaginous callus is an inseparable part of the healing of fractures of long bones, it is very thin, more fibrous, and less cartilaginous when the bone ends are compressed together between transfixation pins and turnbuckle clamps.

When the defect is large, a model of connective tissue and fibrocartilage is formed, similar to the cartilage model of embryonic osteogenesis. The conditions giving rise to cartilage, instead of bone, in the fracture callus are not known. Age, regional factors, and species differences, however, influence the capacity of the individual to produce cartilage. Growing animals produce more cartilage than adults; they frequently form islands of cartilage concurrently with formation of trabeculae of new bone. Long bones formed as cartilage models in fetal life invariably produce cartilage in a fracture callus. Flat bones formed by intramembranous ossification heal without the appearance of cartilage; surgical defects in the calvarium heal along the edges of the bone but osteogenesis in this area of the skeleton in adult life is indolent and fails to produce complete filling.

Rats and rabbits have a greater capacity to form cartilage than do guinea pigs, dogs, and man; motion and function cause an increase in the amount of cartilage. Low oxygen tension and low blood supply in the fracture gap are said to promote chondrogenesis; the evidence is scanty.

The significance of cartilage in fracture callus merits further study. While chondrogenesis in mammals is relegated to an accessory or nonessential part of bone repair, it plays a dominant role in lower animals. In reptiles, if the metabolic processes are slowed by

low body temperatures, cartilage transforms directly into bone. Similar phenomena can be seen in chondro-osteoid in rachitic mammals and in various pathologic lesions in man. Lacroix believes that cartilage acts as an organizer and that it not only elaborates the skeletal tissues but also arranges them in their normal order.

OSTEOGENESIS

New-bone formation begins in young individuals on the inner and outer surfaces of the damaged bone as early as 48 hours after injury. It arises from periosteum and endosteum, at some distance from the fracture line, and grows toward the fracture gap, enveloping and replacing the fibrocartilaginous callus. This bone originates from cells with inherited osteogenetic potency; as it advances into the fibrocartilaginous callus, perivascular connective tissue cells are drawn into osteogenesis and are transformed into osteoblasts (Fig. 4, chap. iii).

Bone does not form at random within the fibrocartilaginous callus but grows by extension of periosteal and endosteal new bone. The tendency has been to regard the fibrous connective tissue and cartilage of the callus only as a model or scaffold for osteogenesis, to be invaded and replaced by bone. As in the case of embryonic cartilage models of bone, however, it is reasonable to regard the cartilage as playing an active, rather than a passive, role in osteogenesis and to assume that the cartilage exerts its influence through the mechanism of *induction*. This concept is strengthened by the observation that when autogenous, homogenous, and even devitalized transplants of fibrocartilaginous callus are made to the anterior chamber of the rat's eye, they lead to production of new bone.

In both the embryonic cartilage model and the fibrocartilaginous callus, connective tissue cells that otherwise would not have exhibited osteogenetic potencies are induced to do so by the proximity of bone or of cartilage. The formation of new bone may thus require the participation both of cells predisposed to exhibit osteogenetic activity and of others that do so less readily, but enter into the process under the influence of induction. The end result is replacement of the fibrocartilaginous callus by bone, bony union of

the fracture fragments, and reorganization of the callus with removal of excess bone.

When a polyethylene tube is implanted across a gap in the shaft of the fibula of a young animal, the plastic prevents sealing of the bone ends. The events of repair are separated in time and space as the cells migrate into the tube. Osteogenesis is prepared for and initiated during a preliminary stage of migration and mitotic division of reticular cells. Cartilage, and to some extent bone, develop in a fluid- and fibrin-filled space. The conditions for survival of the cells in this environment are limited and temporary. Resorption of calcified cartilage or bone, however, provides the conditions for continuation of osteogenesis; resorption can occur with or without the action of osteoclasts. The sequence of events within the walls of the polyethylene tube is: (1) outgrowth of capillaries and mitotic division of reticular or endosteal cells; (2) differentiation of chondroblasts and osteoblasts; and (3) alternating phases of resorption and reconstruction of bone. Guided by the plastic tube, osteogenesis occurs across a gap that could otherwise not become bridged by new bone.

ROLE OF PERIOSTEUM

The external callus of the healing fracture develops from periosteum. In adults this membrane is morphologically indistinguishable from ordinary dense connective tissue. In young growing individuals and in adults after a fracture, the periosteum consists of an inner layer of proliferating osteoblasts and an outer layer of fibrous connective tissue, including fibroblasts. The osteoblastic layer is activated by the injury to the underlying bone, and it deposits new bone until the outer layer is separated from the cortex by a spindle-shaped mass, enveloping the ends of the bone and forming the external bony callus.

The contribution of the periosteum to the repair of bone is of major importance. An intact periosteal tube is capable of regenerating a large segment of a rib or the entire diaphysis of a long bone. In very young individuals this regeneration is complete. In adults, with less active periosteum, the end result is imperfect and inadequate.

These observations indicate that the osteogenetic potency of the periosteum is derived from and is somewhat dependent upon a close *and continuous association with bone. If the periosteum is excised,* the body can replace it with a new connective tissue membrane. This applies itself closely to the shaft and can function as well as the original periosteum in producing an external callus for repair of a fracture. Parts of the skeleton, such as the proximal portion of the neck of the femur, the patella, and other locations from which the periosteum disappears after growth is complete, must depend entirely upon endosteal bone formation for repair.

The extremes can be seen in the healing of fractures when the marrow cavity is obliterated by a steel rod for intramedullary fixation and the repair of bone occurs almost entirely by periosteal activity. The regeneration of bone is rapid and efficient where the periosteum is well developed and can re-form when damaged. The repair of bone is slow where the periosteum is vestigial, inactive, absent, or not regenerated.

SPECIES DIFFERENCES

The organization of fracture healing is the same in all vertebrates. Some differences in the repair described in mammals can be seen in birds, reptiles, and amphibia. Bone repair has been little studied in fishes. Removal of fibrocartilaginous callus is extremely slow in cold-blooded animals; some of the cartilage that is uneroded by blood vessels appears to transform directly into bone. Pritchard and Ruzicka described removal of cartilage by chondroclasts in the lizard. There was virtually no calcification of fibrocartilaginous callus, and alkaline phosphatase activity of the tissue was always extremely low in the frog. The chief source of new bone, however, in all species is periosteum and endosteum.

BLOOD SUPPLY

The vascular network of the callus is an entirely new growth of small arteries, capillaries, and veins, arising as branches of blood vessels of surrounding muscles, periosteum, and bone marrow. As is the case in the granulation tissue of any healing wound, the new blood vessels, including the smooth muscle of the arteriolar walls, *originate, at least in part, from differentiation of perivascular con-*

nective tissue cells. The magnitude and complexity of the system correspond to the size of the callus. In specimens injected with dyes or radio-opaque solutions it may be demonstrated that callus is abundant and healing is rapid in areas with large collateral circulation, such as the metaphyses of the long bones. It is slow in areas, such as the head of the femur, where the blood supply is limited to terminal arteries.

The circulation in the midshafts of long bones is reduced in adult life to a volume barely adequate for compact bone and insufficient for rapid regeneration of large areas of damaged bone. Since callus is new growth with a new system of vessels, the vascular bed develops in proportion to the proliferative response of the bone tissue and is usually adequate to accomplish the healing of a fracture. After the fracture is united, there is regression of connective tissue, absorption of excess cartilage and bone, and disappearance of the entire vascular system of the callus. This is a characteristic of the healing of fractures that distinguishes callus from neoplasms of bone and from such conditions as myositis ossificans.

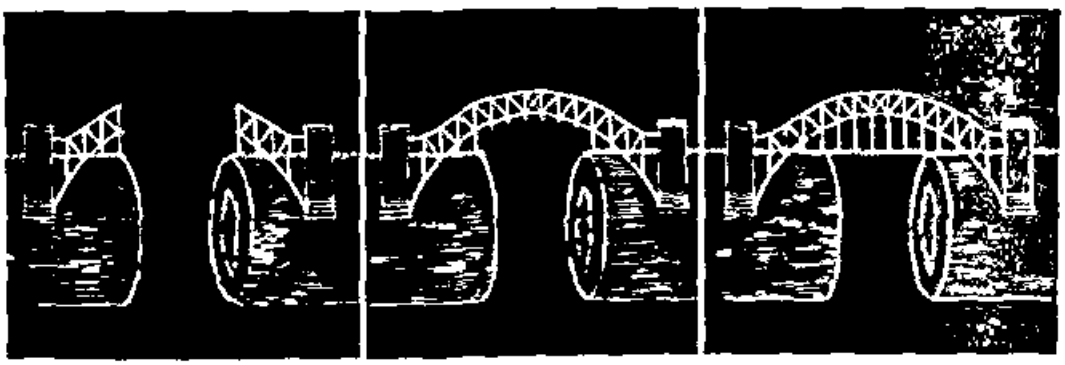
UNITING OF FRACTURE

The bridging of a fracture by new bone, as seen in a sagittal section, occurs according to a plan resembling that of a fixed-arch bridge. The principle is that of cantilevering out and is frequently seen in contemporary architecture. The new bone grows out upon the surface of the model and envelops the fibrocartilaginous callus, to form an arch of new bone over the fracture gap. Like ribs and spandrels let down from the arch of a bridge to suspend the deck, the new bone grows through and replaces the cartilage centripetally toward the fracture gap. Finally, the deck is laid down between the fracture ends and provides for permanent union. The healing fracture differs from a fixed-arch bridge, in that when the fracture is healed the superstructure disappears, leaving only the bone required for union of the fracture ends (Figs. 31, 32, 33).

The shape of the callus and the volume of tissue required to bridge a fracture depend upon the amount of bone damage and displacement. The healing time is directly proportional to the total

volume of damaged bone and the breadth of the fracture defect. In impacted fractures a microscopic plate of fibrocartilage is formed at the line of the injury and is replaced by new bone within a few weeks. In displaced fractures the defect becomes filled with a great mass of fibrocartilaginous callus and fibrous tissue, requiring months or years to be replaced by new bone.

The healing time of each bone in the body is predetermined and related to regional conditions. Accurate estimates are available for fractures in the skeletons of the rat and of man. The bones of the upper extremity, in general, heal more rapidly than those of the lower extremity. In man, in fractures with the bone ends in contact, humerus and forearm bones unite in three months. The femur and tibia usually require six months. In rats the same fractures heal in four and eight weeks. Spiral fractures heal more rapidly than transverse fractures. Separation of the fragments, even when slight, will



greatly increase the healing time in fractures through dense compact bone in adults. Young individuals produce more callus and heal fractures faster. Double fractures of the midshaft require sequences of healing following the direction of flow of the nutrient arteries. In the femur the distal fracture line unites first and the proximal later. In the tibia the proximal fracture line unites first and the distal later; it may even fail to unite if the period of immobilization is insufficient.

SYSTEMIC FACTORS

The local and the systemic factors in bone repair are interdependent. The minimum subsistence level of nutritional factors essential for life is apparently all that is required to heal a fracture. The callus appears to hold the highest priority on all tissue-building materials in transport. Multiple fractures in rats heal without delay. The animal loses a large part of its body weight, owing to loss of muscle tissue. Metabolic balance studies with nitrogen,

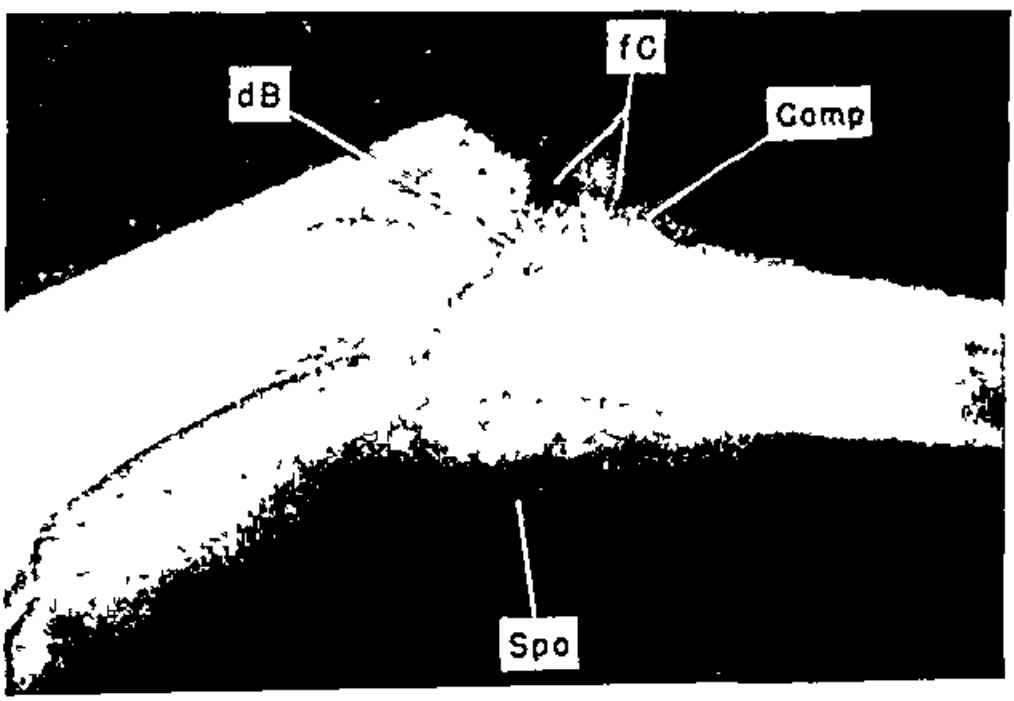


FIG. 32.—Roentgenogram of a fracture of the femur after 85 days of healing, showing a spindle of bony callus inclosing the fracture site but not yet joining the cortical ends, which are still separated by fibrocartilaginous callus (*fC*). *Spo* indicates spongiosa, *Comp*, compact bone, and *dB*, an isolated fragment of dead cortical bone. (From Fig 25, Urist and Johnson, J. Bone & Joint Surg., 25:32. Reproduced by courtesy of the publisher.)

phosphorus, potassium, and sulfur indicate that muscle tissue catabolism supplies all the materials needed for building bone matrix. The body elects to catabolize muscle to meet the exigency of the moment when there is need for rapid construction of new bone.

CALCIFICATION OF CALLUS

The growing callus is calcified in the same way as are cartilage and bone in other parts of the skeleton. Calcification occurs only in a specially prepared matrix. New bone ordinarily calcifies in the callus as soon as it is formed, provided that sufficient concentrations of calcium and phosphate ions are present in the blood plasma. In healing fractures in rickets, calcification and ossification can be entirely separate phenomena. In rickets, in the early stages of frac-

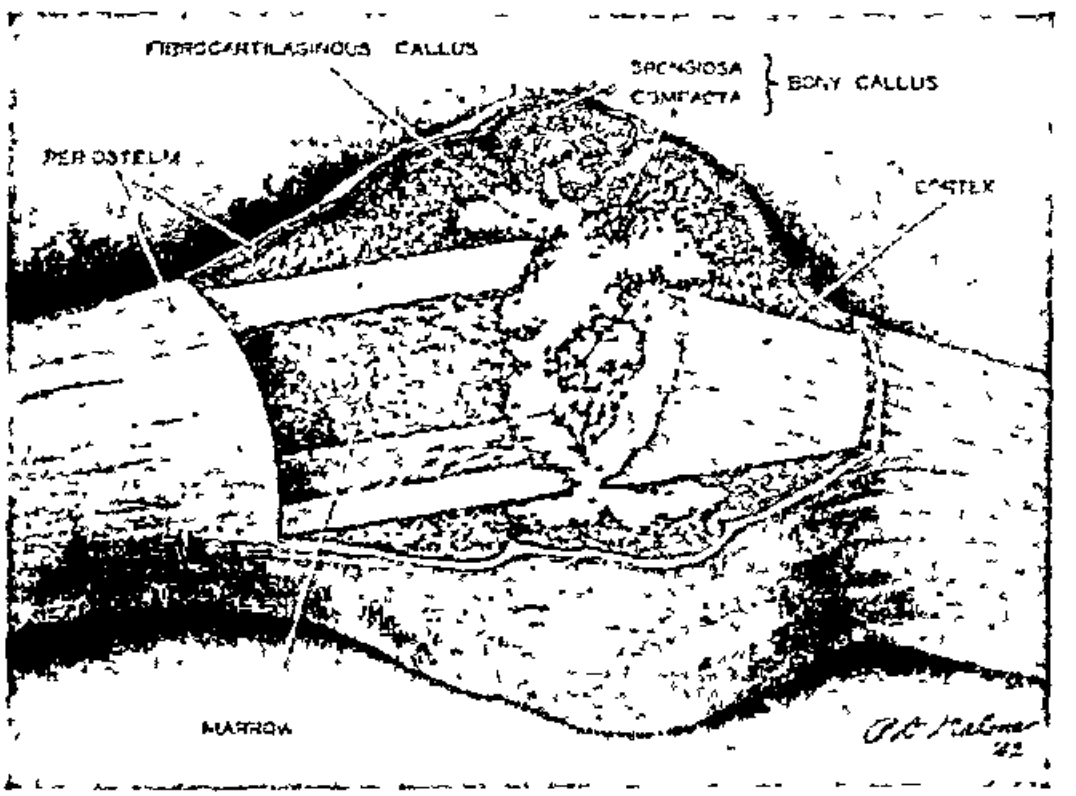


FIG. 33.—Artist's reconstruction of the fracture site, drawn from a clay model of the roentgenogram in Fig 32. Histologic sections of various parts of the callus were placed on the appropriate plane of the sectioned surfaces and reproduced as magnified. The cartilage, fibrocartilage, and dense fibrous tissue of the fibrocartilaginous callus are seen as a homogeneous, glistening, white scar between and about the fracture ends. (From Fig. 27, Urist and Johnson, J. Bone & Joint Surg., 25:35. *Reproduced by courtesy of the publisher.*)

ture healing, the new bone is laid down as osteoid tissue without any calcium salts. The fibrocartilaginous callus also fails to calcify and leads to the formation of chondro-osteoid rather than bone. The sequence is the same as in the rachitic metaphysis, and it results in a disorganized mass of callus and a measurable delay in union of the fracture.

New-bone formation advances into the callus along a broad front from each side and replaces fibrous connective tissue, fibrocartilage, or hyaline cartilage alike as it moves across the fracture gap. Wherever a calcifiable tissue is encountered, calcium is deposited at the line of contact with the osteogenetic tissue or bone. In the early stages of healing, particularly in young individuals, the rate of calcification may lag behind the rate of new-bone formation. The bone trabeculae formed under these conditions have thin osteoid borders. Such small areas of uncalcified cartilage and bone in the callus are encountered in patients on standard or average hospital diets and do not indicate rickets. The levels of serum calcium, inorganic phosphorus, and alkaline phosphatase are not appreciably altered during fracture healing.

X-ray diffraction patterns of the mineral in callus are typical of hydroxyapatite. The orientation of the crystals as observed by X-ray diffraction and by polarization microscopy is in the long axes of the collagen fibers. Densitometric measurements reveal a relatively slight increase in deposition of bone mineral after the initial calcification is complete. The content of organic material, as observed by microinterferometry upon decalcified sections of bone, is the same in partially mineralized and fully mineralized bone.

TRACER STUDIES OF FRACTURES

Radioisotopes of calcium, strontium, phosphorus, and sulfur are avidly removed from the circulation by growing callus. Uptake of S^{35} occurs with synthesis of chondroitin sulfate and other mucopolysaccharides in the matrix of fibrocartilage and hyaline cartilage, four to twenty-one days after the injury. Ca^{45} or Sr^{90} is deposited in the calcifying new bone matrix, and in the zone of provisional calcification during endochondral ossification at from four

to one hundred and twenty days of healing. The areas of uptake of P^{32} correspond closely to those of Ca^{45}; it is deposited in a more concentrated form as hydroxyapatite than as organic phosphorus. Because of the relatively low concentration of sulfated mucopolysaccharides in bone tissue, less S^{35} can be found in bone matrix than in cartilage matrix; the S^{35} content of the callus is accordingly markedly reduced in the course of the replacement of the fibrocartilaginous callus by bone.

The time relationships and sequence of events in bone repair may be observed by administration of tracer doses of Ca^{45}. Preliminary repair, with callus formation and calcification, occurs within a few weeks after trauma, while resorption, reconstruction, and redistribution of the bone tissue continue for months and sometimes years. For the most part, however, increase in mineral density, hardness, and weight-bearing capacity, develop together. In this respect the bone tissue forming in the callus is similar to that in the skeleton as a whole.

MINERALS, VITAMINS, AND HORMONES

The search for a substance which might stimulate healing of fractures has motivated study of the effects of minerals, vitamins, and hormones upon callus formation. Thus far nothing specific has been found either to suppress or to stimulate bone repair. The situation is much the same as with the present state of knowledge of the physiology of wound healing in general. Certain phases of the formation of fibrous connective tissue are affected by systemic factors, such as vitamin C or cortisone. By subjecting experimental animals to deprivation of vitamin C or overdosage of cortisone, it is possible to produce a poor quality of callus but eventually the fracture will heal.

DELAYED OR NON-UNION OF FRACTURES

A large part of the literature on bone regeneration has been written by physicians who hoped to find an answer to the problem of slow-healing fractures in man. The emphasis of textbook dogma on the subject has been upon mechanical causes and mechanical

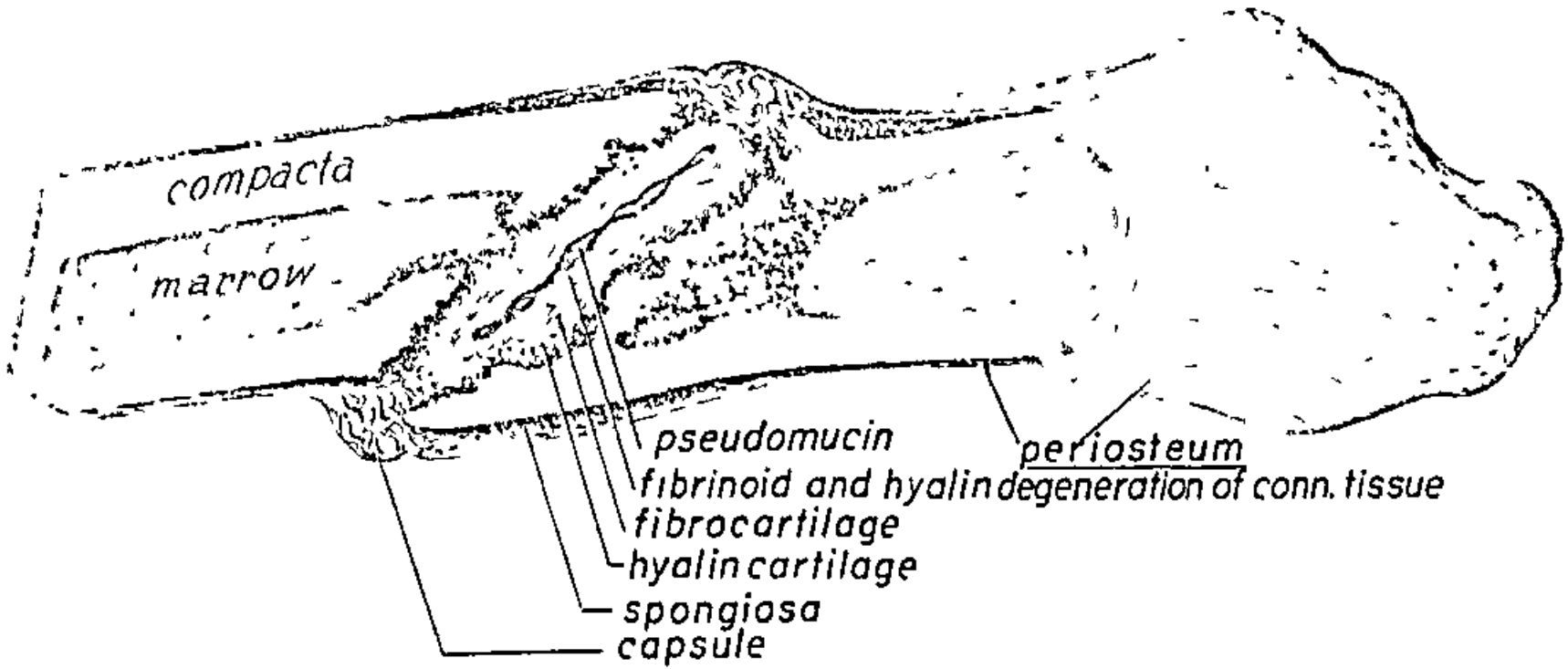


FIG. 31.—Artist's drawing to illustrate the gross and microscopic anatomy of the callus of an ununited fracture at 2 years and 9 months of healing, reconstructed from small samples of tissue obtained from all parts of the fracture site and the bone ends of the tibia of a man thirty-five years of age. (From original drawing of Fig. 7-G, Urist, Mazet, and McLean, J. Bone & Joint Surg. 36A:949. Reproduced by courtesy of the publisher.)

treatment. Observations on callus formation, using histochemical techniques, suggest that non-union is not the absence of healing per se but the failure to set up an induction system for new-bone formation in the area between the bone ends. The adult human tibia, the bone which offers one of the great challenges to fracture treatment, provides the material most suitable for study of slow-healing fractures.

When bone repair is observed in a consecutive series of control cases compared with matched cases of ununited fractures, the healing time is always proportional to the total length of the damaged area of bone and the breadth of the fracture gap. The adult human tibia produces about 1.0 cm. of bone per year—0.5 cm. from each bone end. After eighteen months the rate of proliferation subsides to the normal slow rate of turnover of tibial bone tissue. If the area of bone damage and fracture gap exceeds 1.0 cm. and if the fracture does not unite within eighteen months, the interior of the callus may develop an amorphous center, with fibrinoid and hyaline degeneration of connective tissue (Fig. 34).

FIBRINOID

Fibrinoid forms in the interior of the callus if the fracture is extensive, complicated by infection, or difficult to immobilize. The occurrence is similar to that in chronic adventitious bursitis, except that the lesion develops in the space between the bone ends rather than in the subcutaneous region over a bony prominence. The formation and composition of fibrinoid indicate that it is a by-product of trauma and repair.

Morphologically, fibrinoid is a mass of collagen and ground substance, in all stages of degradation. It is like fibrin, as its name implies, but it is not fibrin. It is acellular, homogeneous, highly refractile, and acidophilic. It has the staining reactions of mucopolysaccharides; i.e., it stains metachromatically with toluidine blue, pink with PAS reagents, and a mixture of orange, brown, yellow, and blue with phosphotungstic acid and hematoxylin. X-ray diffraction and the electron microscope reveal disoriented collagen fibers in the early stages and only amorphous material remaining in the later stages of fibrinoid degeneration.

After any mechanical disturbance of the fracture site, if it contains fibrinoid, the amorphous material may split apart and produce a cavity filled with mucinous fluid, in the center of the callus. Fibrinoid imbibes water, and the internal pressure created by osmosis may be sufficient to break metallic appliances used for internal fixation and to refracture necrotic bone. If motion and friction are not controlled, fibrinoid degeneration continues indefinitely, and a permanent pseudarthrosis may develop. All the new bone formed in the area then becomes converted into compact bone *inside and around the cortical ends*, but not across the fracture site, and this forms articulating false-joint surfaces.

Immobilization acts as a deterrent to the formation of fibrinoid and permits refilling of the defect with new fibrocartilaginous callus. By this means the degradation can be reversed up to eighteen months after injury, and bone repair can progress simply by prolonged immobilization. After eighteen months, excision of the fracture site and close coaptation of the bone ends is more likely to produce union.

Injury to blood supply has been regarded by many writers of many years past as *the main cause of failure of bone repair. This* generally occurs in adult life in parts of the skeleton where there is inadequate collateral circulation, dense avascular compact bone, arteriosclerosis, or excessive splintering of cortex due to osteoporosis.

A bone graft of any kind produces a new proliferative response from the bone ends and new-bone formation by induction across the fracture gap. Homogenous and autogenous bone appear to be equally effective in most cases. Inlay, onlay, and intramedullary grafts are all capable of producing union in the majority of cases. The success of the operation depends upon the proliferative response of the bone ends rather than upon the technique or the *material employed. These observations suggest* that the mechanical treatment of fractures can be overemphasized and that early open operations and metallic internal fixation should be used only in unusual circumstances. More reliance should be placed upon the primordial power of the human skeleton to regenerate injured and missing substance.

CHAPTER XV

Pathologic Physiology of Bone

We shall restrict our consideration of pathologic conditions to a few of those illustrating the effects of disturbances of the normal physiology of bone. These will include abnormalities in the formation of bone matrix; defective calcification of matrix; and the effects upon bone of hypersecretion of the parathyroid glands. They will exclude all new growths and infections of bone; a variety of polyglandular syndromes; and most systemic or localized affections of bone whose genesis is not clearly understood.

1. Atrophy, Osteoporosis, and Rarefaction of Bone

Strictly speaking, *atrophy* of bone should refer to loss of substance or of volume; *osteoporosis* to an increased porosity, i.e., to a decrease in the hard portions of bone substance in favor of a relative increase in the soft portions; *rarefaction* to decreased density, i.e., to a decrease in the weight per unit of volume. Since roentgenograms do not distinguish clearly between conditions resulting in a decrease in the density of the shadows cast by bones and since the nature of the condition is often obscure, both in the clinic and at autopsy, the tendency is to use the three terms interchangeably. Excluded from these terms, however, are rickets and osteomalacia, both being differentiated from osteoporosis or rarefaction by failure of calcification of the matrix, the production of which is not interfered with; also excluded is the softening of bone by increased resorption resulting from hyperparathyroidism.

In our opinion *atrophy* is the term that most nearly describes the condition characterized by deficient formation of new bone matrix, and we have used this as a general designation. Occasionally,

however, it is difficult to avoid the use of one of the other terms, especially in connection with the descriptions of others, e.g., the "osteoporosis of Cushing's syndrome."

Atrophy of bone is unique and unlike atrophy of soft-tissue structures. In soft tissues, such as skin, muscle, or liver, atrophy produces a reduction in the size as well as the number of cells and results in shrinkage of the external dimensions of the whole organ. Atrophy of bone occurs without a corresponding change in the volume or external dimensions of the bone, but the mass of bone tissue may be reduced as much as 75 per cent. The internal architecture of the bone gradually becomes attenuated and finally disappears. The process is seen in its most typical form in the bones of paralyzed limbs or parts immobilized in casts for long periods of time; these exhibit the *atrophy of disuse.* The first change is a diminished mass of bone tissue per unit area of the bone, recognizable by microscopic examination, at autopsy, or in roentgenograms.

An atrophied bone is everywhere brittle and of a more spongy consistency than normal. In cross-section the cortex is thin. The periosteal surface is smooth and unchanged, but the intramedullary surface is composed of a yellow, fatty, cancellous bone tissue. In roentgenograms the bone tissue casts a homogeneous diffuse shadow, with the individual trabeculae widely separated, thin, and difficult to outline.

The microscopic structure of the cancellous bone tissue consists of very thin trabeculae, branching, deficient in length, and with smooth surfaces completely devoid of osteoblasts. The bone marrow is uniformly fatty, fibrous, and hypoplastic. A loss of 24–30 per cent of the bone salt is necessary to produce an appreciable change in roentgenograms of the bone; in severe cases of atrophy, with extreme roentgenographic changes, more than 50 per cent of the mineral of the skeleton may be lost (Fig. 35).

Bone atrophy may be systemic, regional, or local. The use of the term *osteoporosis* usually refers to a systemic condition, in contrast to the local atrophy of disuse. The terms *senile osteoporosis* and *postmenopausal osteoporosis* add to the confusion in the literature on this subject. The physiologic atrophy of the bone tis-

FIG. 33.—Normal vertebral body (*above*) and osteoporotic vertebral body (*below*), both after maceration. Insets are radiographs of the two specimens. The system of thick trabeculae and small transverse plates, seen in the normal bone, is reduced, in the osteoporotic bone, to a delicate web of thin struts, with very few plates of bone and with a reduction of mass. ×4·5 (From originals of Figs. 92 and 93, Collins, Modern trends in diseases of the vertebral column [London: Butterworth and Co., Ltd., 1959], pp. 115 and 116. Reproduced by courtesy of the publishers.)

sue in old age differs in no way, either roentgenographically or microscopically, from the atrophy of disuse or osteoporosis.

It is usually stated that atrophy is the result solely of deficient formation of bone, in the presence of a normal rate of destruction of the tissue. The rapidity with which the atrophy of disuse develops in an immobilized limb has suggested that an increased rate of destruction may also be a factor in its production. This view is supported by the observation of calcium balance and urinary excretion during immobilization. Normal young men, immobilized in plaster casts, lost 1–2 per cent of their total body calcium within six to seven weeks. A growing boy, placed in a partial body cast for a fracture of the neck of the femur, suffered extreme atrophy of the immobilized parts of the skeleton, with hypercalcemia and increased excretion of calcium in the urine. The occurrence of kidney stones in patients immobilized in casts is frequently observed; this is a sequel of the increased loss of calcium by excretion in the urine.

2. Problem of Osteoporosis

In osteoporosis, as it is commonly seen in the aged, but sometimes also in younger men as well as women, 50–75 per cent of the internal architecture of the ribs, vertebrae, pelvis, and the necks of the femurs may disappear. The condition is generally discovered by fracture either after a trivial injury or no actual injury at all. Radiographs of the spine reveal ballooning of the intervertebral disks, thinning of the cortex, and accentuation of the vertebral trabecular markings. The bones of the extremities are affected relatively late in the course of the disorder. Microscopically, the bone tissue that is present consists of trabecular and haversian bone enveloped in fibrous and lipoid connective tissue. The cortex becomes light in weight, hard in substance, and very brittle. Microradiographs show fully calcified lamellae of old bone of both high and low density and vascular channels containing plugs of amorphous calcium deposits. Bone accretion as determined by tracer studies may be normal, but in some cases a lower percentage of radioactive

isotopes of calcium and phosphorus is deposited and retained in osteoporotic patients than in normal subjects.

A satisfactory experimental laboratory mammal is yet to be found for study of osteoporosis. A spontaneous form of osteoporosis, related to genetic factors but in some respects resembling the disorder in man, is found in the White Leghorn hen bred for heavy egg production. This condition is known in the poultry industry as *cage layer fatigue*, is aggravated by confinement in close quarters, and is improved by exercise. Osteoporosis, experimental as well as clinical, is characterized by normal levels of serum calcium, phosphorus, and alkaline phosphatase. Mineral, vitamin, and general nutritional status are usually within normal limits. Metabolic balance studies upon young subjects with rapidly progressive osteoporosis reveal daily losses in calcium, phosphorus, and nitrogen in the urine and feces in excess of the amounts in the dietary intake. Aged individuals with slowly progressive osteoporosis, commonly seen with fracture of the neck of the femur as the presenting symptom, are usually not in negative calcium, phosphorus, or nitrogen balance. The pathogenesis appears to be a depressed or very low rate of accretion, associated with high or normal rate of resorption.

The etiology of osteoporosis is rarely demonstrable in man. Patients with Cushing's syndrome are exceptional; the effects of exogenous hypercortisonism are seen more frequently, both in man and in experimental animals, and are not uncommon in children. Proliferation of connective tissue cells, including osteoblasts, is inhibited by corticosteroids; large amounts of calcium are unabsorbed. On correction of hypercortisonism, young individuals may regain bone structure by appositional new-bone formation, but recovery of the original mass of skeletal tissue has not been observed in adults.

During the past decade the view that osteoporosis is the result of an imbalance between gonadal and adrenocortical hormones has gained wide acceptance and has dominated the therapy of the disorder. This view, advanced by Albright and Reifenstein, has recently been modernized by Reifenstein, in the form that the anti-

anabolic effects of a normal output of corticosteroids may overcome the anabolic effects of the reduced postmenopausal output of estrogens, the result being a negative calcium and nitrogen balance and osteoporosis.

An older view is that osteoporosis results from a deficient calcium intake. Nordin, supporting this concept, holds that osteoporosis may be associated either with low intake, insufficient absorption, or high excretion of calcium. Whedon and his associates have observed the influence of accompanying debilitating disease, immobilization, inadequate intake of minerals, and treatment with an intake of two or more grams of calcium daily; they report retention of as much as 200 mg. per day in osteoporotics sixty years of age, as well as in normal control adults. A protein starvation theory, citing the effects of protein deficient diets or gastrointestinal disorders, is based upon the similarity of bone changes in hunger osteopathy and in osteoporosis.

Except for the osteoporosis of hypercortisonism, endogenous or exogenous, the etiology is ill defined. One may generalize by saying that calcium deficient diets, castration, hypercortisonism, hyperthyroidism, or weight loss from starvation or debilitating disease, all may accelerate the progress of the disorder in individuals predisposed to develop it in old age. This supports the view of a non-specific aging, suggested by the higher incidence of the condition in the aged population. Young patients with osteoporosis have the external appearance of individuals ten to twenty years older than their chronological age. Urist and Vincent have observed a decline in the excretion of 11-deoxy-17-ketosteroids in young women with osteoporosis; this was observed also after age seventy in women without osteoporosis and was attributed to the non-specific effects of aging upon the adrenal cortex.

As has been noted, *osteoporosis in adult life is refractory to treatment* and irreversible with respect to recovery of normal density of the bones. Until the etiology is known or a specific stimulant for osteogenesis is found, treatment with high calcium diets or sex hormones, or with combinations of the two, will continue to be of limited value.

3. Rickets and Osteomalacia

Rickets

Rickets may be defined as a failure of calcification to keep pace with the growth of bones. This implies that rickets is a disease of the growing child; a somewhat similar pathologic condition seen in the adult, under extreme deprivation of vitamin D and of minerals, is known as *osteomalacia*.

One of the most constant and most characteristic sequelae of what may be called a functional deficiency of phosphate—generally as the result of a low intake of vitamin D—is the lowering of the concentration of inorganic phosphate in the blood plasma. The simplest conception of rickets is that which attributes all the symptoms and findings in this disease to the lowered plasma phosphate.

In experimental animals fed with rachitogenic diets, the earliest evidence of rickets is failure of calcification at the epiphyseal-metaphyseal junction and the appearance of uncalcified osteoid tissue on the surfaces of growing trabeculae of bone. The failure of calcification, particularly in the epiphyseal cartilage, is responsible for the subsequent pathologic changes, all of which result from the growth and accumulation of cartilage and of osteoid tissue.

Vitamin D–Resistant Rickets

There is a series of disorders characterized by the skeletal manifestations of rickets or osteomalacia but resistant to therapy with vitamin D. Various forms have been described; for the most part they have in common a disturbed function of the renal tubules, resulting in a relative phosphaturia with a lowering of the serum phosphate level, conducive to a failure of calcification. At the other end of the scale there is a condition known as *idiopathic hypercalcemia*, believed to be a manifestation of hypersensitivity to vitamin D. Fanconi has described the variations in sensitivity to vitamin D, and his name is prominently associated with the clinical manifestations of these variations. Harrison has also described the varieties of rickets and osteomalacia associated with hypophosphatemia, including the Fanconi syndrome. Engfeldt *et al.*, who have reported microradiographic studies of the bones in refractory

rickets, have observed that the structural features differ from those in ordinary rickets and have concluded that the condition is a genetically determined entity. Treatment with massive doses of vitamin D is commonly employed, with variable success. Any improvement so obtained has been attributed to the calcemic effects of the large doses of vitamin D; this treatment does not influence the morphology and does not cure the disease.

OSTEOMALACIA

Osteomalacia is the adult form of rickets. Since it involves only the bones of adults, in the absence of the growth apparatus of infancy and childhood, it is characterized pathologically by failure of newly formed bone matrix to calcify. In borderline cases osteoid margins on otherwise calcified trabeculae of bone may be demonstrated, advanced cases are notable for softening of all the bones and consequent deformities.

Osteomalacia has been known for centuries in India and in the Middle East. It is now rare in Europe and America, but occurred in combination with osteoporosis in some of the European countries during World War I, associated with pregnancy and dietary privations. Classical osteomalacia has been carefully studied in China, where it has assumed endemic proportions, resulting from lack of vitamin D, combined with a very low intake of calories, protein, calcium, and phosphorus. Even under these extreme conditions it is rare except as a sequel to pregnancy, with its drain on the skeletal system of the mother. It occurs, but very rarely, in males.

Several conditions occur, also rarely, in which the bones have the roentgenographic and microscopic characteristics of osteomalacia, but without the usual etiologic factors. The most common form is that which accompanies an excess of fat in the stools, with failure of absorption of vitamin D. Renal acidosis may also be accompanied by softening of the bones; this condition is distinct from renal rickets, which is a form of secondary hyperparathyroidism.

In both rickets and osteomalacia the serum calcium is usually maintained at a normal level; the inorganic phosphate of the serum is low; the alkaline phosphatase is high. Secondary enlargement of the parathyroid glands is common in both disorders.

4. Hyperparathyroidism

Primary Hyperparathyroidism

Primary hyperparathyroidism is the idiopathic form of hyperparathyroidism. In most instances there is a single adenoma; more rarely there are multiple adenomata; carcinoma is still less common. Idiopathic hypertrophy, in which the increase in the size of the glands is in part attributable to enlargement of the cells, also occurs.

The manifestations of primary hyperparathyroidism are the result of hypersecretion of the parathyroid hormone; they are independent of the nature of the disorder of the gland. The most common and almost pathognomonic finding is that of elevation of the serum calcium level. The increase in concentration of the total calcium is divided between calcium ions and undissociated calcium proteinate, distributed in accordance with the law of mass action. With few exceptions, increase in the calcium ion concentration of the serum is diagnostic of primary hyperparathyroidism, especially if accompanied by a normal protein and low phosphate level. Any finding of a serum calcium level above 11.0 mg. per 100 cc (2.75 mM per liter) or a calcium ion concentration above 5.5 mg. per 100 cc. as estimated from serum calcium and serum protein (Fig. 25) should arouse suspicion. Serum calcium values above 15.0 mg. per 100 cc. are rare, but may occur.

Although hyperparathyroidism first made itself known by its production of skeletal disease, skeletal changes are by no means an essential part of the findings. Hypercalcemia leads to increased excretion of calcium in the urine; this, in turn, leads to the formation of kidney stones. The possibility of hyperparathyroidism is brought to the attention of the physician as often by kidney stones as by symptoms and signs originating from the skeleton.

The gross skeletal changes in advanced cases are those of softening of the bones, with consequent deformities and fractures. The diagnosis is often suggested by the roentgenographic findings of generalized decrease in density of the bones, often accompanied by cysts and tumors. The microscopic findings are those of greatly increased resorption of bone, with numerous osteoclasts. These are

accompanied by increased osteoblastic activity, indicative of attempts to repair the damaged trabeculae of bone. Attempts at repair also result in an excess of fibrous connective tissue; this has led to use of the term *osteitis fibrosa* to describe the pathologic changes in the bones. Such bone matrix as is present, old and new, is calcified; it is not correct to refer to the changes in the bone as decalcification.

In many cases of hyperparathyroidism, certainly in borderline cases, no bone changes can be found. They may also be prevented by providing a liberal intake of calcium. When bone changes are present, the serum phosphatase level is elevated; when they are absent or are prevented by increased calcium intake, the serum phosphatase is within the normal range.

SECONDARY HYPERPARATHYROIDISM

Hyperplasia of the parathyroid glands, accompanied by increased secretory activity, occurs under the following conditions: rickets or osteomalacia; pregnancy; renal insufficiency, associated with retention of phosphate; and calcium deprivation. All these conditions predispose toward low serum calcium; hyperplasia of the parathyroid glands is compensatory; hypersecretion rarely leads to elevation of the serum calcium above normal values. Erdheim's description of enlargement of the parathyroid glands in osteomalacia, in 1907, led to some later confusion between primary and secondary hyperparathyroidism. This was not entirely clarified until after Mandl had performed the first parathyroidectomy, for adenoma of the gland, in 1925.

Of the foregoing conditions, only renal insufficiency leads to enough increased activity of the parathyroids to bring about skeletal changes. These changes, because of their association with glomerular nephritis, have become known as *renal rickets*. The sequence of events, each stage being dependent upon the one just before, is: (1) renal insufficiency; (2) diminished excretion of phosphate; (3) elevation of serum phosphate level; (4) depression of serum calcium level; (5) hyperplasia of parathyroid glands with hypersecretion; and (6) resorption of bone.

increased cardiac output, enlargement of the heart, hypertension, severe arteriosclerosis, and local heat over the involved bones suggest that *multiple arteriovenous shunts may be present inside the* skeleton. However, nothing abnormal has been demonstrated by arteriography. The origin of the bone lesion requires further investigation.

6. Osteopetrosis

Abnormalities of bone causing an increase in the amount of hard tissue at the expense of the soft tissue, without any alteration of the external volume, are generally classified as osteopetrosis or "*marble bones.*" *This disorder is uncommon in man, occurring* chiefly in infants with fatal anemia, and found occasionally in asymptomatic or abortive form in adults, by coincidence, in radiographic examinations. It is sometimes inherited as an autosomal *recessive trait. The original patient, twenty-six years old, was de*scribed by Albers-Schönberg in 1907, but nothing about the etiology has been found by study of hundreds of cases since then. Histologic sections reveal a dense spongiosa and thick cortex with bands of osteoid tissue and densely calcified cartilage. The bone tissue may be woven rather than lamellar in structure. Microradiographs show thick cementing lines and active, but distorted, remodeling processes. Owing to the brittleness of the bones, pathologic fractures are common. The blood is generally normal with respect to calcium, phosphate, and alkaline phosphatase.

Storey has produced a condition simulating osteopetrosis in rats, by intermittent administration of large doses of vitamin D. Alternate cycles of hypervitaminosis D rickets and of recovery result in the association of hypercalcification and of osteoid tissue in the same bones at the same time; the bone changes in some respects resemble those seen in osteopetrosis. The conclusion is that the important mechanism in the pathogenesis of osteopetrosis is an accentuated rhythm of bone changes, similar to those produced experimentally.

There are two living aquatic mammals of the order Sirenia, the manatee and the dugong, that have dense and massive bones, variously characterized as osteosclerosis, pachyostosis, and osteo-

petrosis. The bones of the axial skeleton, as well as of the upper extremities, consist almost entirely of compact bone and of dense cancellous bone, of a fetal type, with no medullary cavities and virtually no hemopoiesis. With the aid of fossil material, representing extinct species, and because of a low basal metabolic rate, together with histologic evidence of low thyroid activity, there have been attempts to account for the skeletal peculiarities on an endocrine basis and to relate them to osteopetrosis as it occurs in man.

Urist and Bechtol have confirmed the findings of Fawcett that the entire skeleton of the Florida manatee, *Trichechus latirostis*, has features in common with osteopetrosis. Osteoclasts are scarce, bone resorption is scanty, and periosteal bone formation, with little or no internal remodeling, continues in certain locations throughout the life of the animal. The thyroid gland is large and contains large follicles filled with colloid. While the bones of the manatee resemble those in patients with marble-bone disease, and while they are suggestive of the changes associated with the anoxia of hypothyroidism, there is no real reason for coupling the evolution of the Sirenia with skeletal disorders in man.

7. Genetic Disorders of Connective Tissue

A group of disorders of the skeleton have in common that they are inheritable and represent inborn errors of metabolism, usually affecting both the collagen and the ground substance of bone. Their manifestations may not be confined to the skeleton, but since the mucopolysaccharides are highly concentrated in cartilage matrix and collagen is so densely packed in bone, the disorders may be brought into sharpest focus in the skeleton.

Hypophosphatasia, described as rickets with a deficiency of alkaline phosphatase is a relatively rare metabolic disorder. It is believed to be inherited through an autosomal recessive gene. The bone lesions result from inability of osteoblasts to elaborate calcifiable organic matrix. The presence of phosphorylethanolamine in the blood and urine results from the inability of the insufficient phosphatase in the body to hydrolyze the ester phosphate linkage.

The *Hurler-Pfaundler syndrome*, also known as *gargoylism* or as

lipochondrodystrophy, an inborn error of connective tissue metabolism, presents an enzymatic defect in which there is accumulation and urinary excretion of large amounts of chondroitin sulfate B and of heparin sulfate. It is characterized by grotesque facies and defects involving the entire skeleton. Genetically two types are recognized: (1) a form transmitted by an autosomal recessive gene, affecting both males and females; and (2) a form transmitted as a sex-linked recessive, affecting only males.

The *Morquio syndrome*, or *chondro-osteodysplasia*, is a condition in which the chondrocytes fail to mature, ossification centers are disorganized, and there are extensive deformities of all the joints. It is characterized by small stature and short mid-phalanges of the hands and feet. There are deficiencies in matrix formation and diminished alkaline phosphatase activity in the epiphyseal plate.

Osteogenesis imperfecta is a connective tissue disease, transmitted as an autosomal dominant, in which the brittle and soft bones are usually the most prominent feature. The organic matrix of the bone is defective; collagen is mainly affected, and it has been suggested that the collagen is anomalous in its amino acid sequence.

The *Marfan syndrome*, in addition to having a predilection for the eye and for the aorta, is also manifest in the skeleton by excessive length of the round bones of the extremities, and by defects in the ligaments and tendons, resulting in loose-jointedness. It is attributed to transmission as an autosomal dominant.

Alkaptonuria, recognized by characteristic changes in color in the urine, owing to the presence of large quantities of homogentisic acid, results from a lack of homogentisic acid oxidase. *Ochronosis*, or a dark pigmentation of cartilage, tendons, ligaments, and of the sclera, is the most notable clinical feature. At autopsy cartilage and fibrocartilage are deeply pigmented; pigmentation may also be observed in tendons and, in advanced cases, in the bones. According to Milch, who has studied the condition in interrelated Dominican families, what has appeared to be a dominant form is probably a recessive form appearing in successive generations through the mating of homozygous affected persons with heterozygous carriers; consanguineous matings increase the likelihood of its occurrence.

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Structure and Chemical Composition of Bone Matrix

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